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Notch Ligand Functionalized Microbeads for T Cell Differentiation of Stem Cells

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Notch Ligand Functionalized Microbeads for T Cell Differentiation of Stem Cells

by

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Dedication

This work is primarily dedicated to my older brother, Mosehaf Taqvi, who passed away Jan. 7, 1990 after a long hard fight with leukemia for 9 years. Mosehaf's love for his siblings and short but accomplished life serve as a lifelong inspiration for me always. My hope is that developments in the field of immunology and biomedical engineering will help patients like him lead a life we are all entitled to live. I also dedicate this to my family, extended family and close friends for all of their love, patience and blessings, without which none of this would have been possible.

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Notch Ligand Functionalized Microbeads for T Cell Differentiation of Stem Cells

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Supervisor: Krishnendu Roy

In recent years, great advances have been made in the field of stem cell differentiation. Seminal insights in the area of developmental biology and tissue regeneration have made *ex vivo* differentiated cells a realistic alternative for transplantation applications. The recent application of these murine-based insights to human systems has paved new paths in autoimmune disease, chemotherapy, and immuno-deficiency research. Such strides would eliminate the hurdles associated with adoptive transfer including limited availability of transplantable cells, site morbidity, difficulties in cell isolation and expansion lag time.

Current approaches in *ex vivo* hematopoiesis and T cell differentiation have begun to explore the effects of biomaterials on differentiation efficiency. These approaches, however, have not fully studied the quantitative effects of biomaterials and their properties on hematopoietic and T cell differentiation generation.

Our goal was to design biomaterials whose properties could be tailored to improve differentiation efficiencies in T cell differentiation. Our work is dedicated to fabricating and characterizing Notch ligand functionalized microbeads for T cell differentiation applications. Our work has shown stable functionalization of Notch ligands on microbeads that can be quantitatively varied to achieve optimal Notch signaling. We have also demonstrated limited cellular toxicity and effective Notch signaling upon exposure to Notch ligand functionalized beads. Finally, we have successfully differentiated T cell progenitors from hematopoietic stem cells using the functionalized microbeads. As a side study, we have fabricated and characterized polymeric PLA scaffolds that were systematically varied and studied for their effects on hematopoietic differentiation efficiency. Insights gained from these studies should provide a better understanding of the microenvironmental signals in hematopoiesis and aid in the development of efficient technologies for the production of hematopoietic progenitors and T cells for therapeutic applications.

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CHAPTER ONE

Introduction: Specific Aims and Overview

1.1 INTRODUCTION

Advances in stem cell transplantations laid down the foundation for a procedure called adoptive T cell transfer where patient T cells are expanded and trained *ex vivo* and infused back into the body to replenish the lymphocyte supply. Although clinical studies have demonstrated promising results in terms of little cytotoxicity and normal T cell function, the challenge to treat severely immunocompromised individuals and at that in a cost-effective and rapid amount of time still remains (Yee 2002).

T cell development takes place primarily in the thymic microenvironment where bone marrow progenitors migrate in through post-capillary venules and undergo a series of cell-cell and cell-matrix interactions that drive the development process forward. Cell surface signals such as Notch ligands, interleukins and growth factors, and chemokines are instrumental in providing the differentiation, proliferation and survival and migration cues necessary for the cells to become fully functional (Ciofani and Zuniga-Pflucker 2007).

Recent methods in ex vivo T cell differentiation have focused on the Notch signaling in the thymus and its apparent dose-dependent role in T cell and lymphocyte development. Early studies exploited the thymic microenvironment for ex vivo generation of T cells using fetal thymic organ cultures and thymic stroma (Weekx et al., 2000; Yeomen et al., 1993). These studies provided great insight in T cell development but were severely constrained due to low cell yield and the complicated and cumbersome nature of the setup (Zuniga-Pflucker 2004). The generation of a Notch ligand transfected

stromal cell line, OP9-DL1, benefited the field greatly and led to the successful differentiation of both human and murine derived T cell progenitors from cord blood, bone marrow and fetal liver sources. These studies have also suggested a threshold for Notch signaling in lymphocyte development where B cells require the least, NK cells require medium levels and T cells require the greatest amount (Ciofani and Zuniga-Pflucker 2007; de Pooter and Zuniga-Pflucker 2007; La Motte-Mohs et al., 2005; Schmitt et al., 2004).

Despite its benefits, the OP9-DL1 cell line method of ex vivo T cell generation suffers from two major disadvantages. Failure to express major histocompatibility complex II proteins have prevented the generation of CD4+ SP T cells. Also, the inherent dependence on the cell line to express the Notch ligand makes quantitative characterization and scaling up difficult (Zuniga-Pflucker 2004).

Protein functionalized microbeads have been extensively used in antigen presentation applications with recent studies showing up to 40 days of successful T cell expansion with anti-CD3 and anti-CD28 antibody functionalized beads (Levine et al., 1997). Microbeads offer a cytocompatible, definable approach to protein presentation without the biosafety concerns of cell surface ligands and the ability to scale up (Trickett et al., 2002). The use of such microbeads for T cell development and Notch signaling applications could lead to a more quantitative understanding of Notch signaling and potentially aid in the production of T cells for therapeutic applications.

1.2 SPECIFIC AIMS

1.2.1 Aim 1: To develop Notch ligand coated microbeads (artificial stromal cells), effectively characterize successful ligand conjugation and demonstrate bead cell compatibility.

We hypothesized that streptavidin-biotin and antibody-antigen interactions are robust enough to present Notch ligands in a controlled manner on a microbead platform in a cellular environment. Furthermore, by presenting ligands on a microbead surface, the effective ligand density the cell "sees" can be tailored both by varying the amount on the surface of the bead and varying the amount of beads delivered.

In this aim, we propose the fabrication of Notch ligand (Delta-like ligand 4, DLL4) coated magnetic microbeads through streptavidin-biotin and antibody-antigen interactions as mentioned above. Optimal conditions for bead fabrication were achieved through in depth studies varying incubation period, bead concentration and ligand concentration for both biotinylated antibody and DLL4 proteins. Surface functionalized DLL4 on microbeads was quantified using commercially available calibrated beads. The stability of functionalized DLL4 on microbeads was also assessed both at 4°C and 37°C in various buffers and media. All studies given above utilized immunofluorescence staining and flow cytometry. Immunofluorescence fluoroscopy and ELISA analysis further demonstrated presence of DLL4 on bead surfaces. These studies were carried out with two different commercially available streptavidin coated magnetic microbeads, one of which utilized a DNA linker to bind the streptavidin coating to the polystyrene bead.

Cellular toxicity of uncoated and functionalized microbeads was also assessed using (3-[4,5-dimethylthiazol-2-yl]2,5-diphenyl tetrazolium bromide) (MTT) assays

where quantitative and temporal effects of Notch ligand coated microbeads on cells were evaluated.

1.2.2 Aim 2: To qualitatively and quantitatively demonstrate successful Notch signaling using Notch ligand functionalized microbeads in a dose-dependent manner.

Our hypothesis for the Notch functionalized microbeads was that the amount of Notch signaling present in a cell could be controlled by varying the ligand density on the bead and/or amount of beads present. We explored our hypothesis through a classic bioactivity assay demonstrating myotube inhibition when Notch signaling is present. Inhibition was confirmed qualitatively through phase contrast microscopy. Additional studies examining intracellular Notch signaling namely staining for the presence of activated Notch receptor (only present when Notch signaling is occurring) were performed using intracellular staining, fluorescence microscopy and flow cytometry. Next, real-time reverse transcriptase polymerase chain reaction (RT-PCR) techniques were utilized to demonstrate quantitative differences in downstream Notch target gene expression upon exposure to various bead to cell ratios. Finally, transient transfection of luciferase under the CBF1 promoter and luminescence evaluation were performed to confirm dose-dependent effects of Notch functionalized beads on cells.

1.2.3 Aim 3: To utilize Notch ligand coated microbeads to quantitatively evaluate the effects of Notch signaling on hematopoietic stem cell (HSC) differentiation towards the T cell lineage.

In this aim, our goal was to study the effect of quantitatively tunable Notch signals on T cell commitment and differentiation efficiency of hematopoietic stem cells. HSCs isolated from mice were cocultured with and without functionalized microbeads at two different doses with appropriate supportive stroma and cytokines and evaluated for T cell commitment efficiency after 1 week of incubation using immunophenotype staining and flow cytometry. Effect of cell-cell contact was also assessed using insert culture.

1.3 OVERVIEW

Chapter 2 provides the necessary background and significance of the project with respect to issues that illustrate how the study aids in the understanding of the field, such as therapeutic relevance, current techniques in providing Notch signaling and ex vivo T cell differentiation. In ***Chapter 3***, the fabrication and characterization of Notch functionalized microbeads are discussed, including the effects of incubation period, bead concentration, ligand density, incubation buffers, temperature and cellular toxicity. ***Chapter 4*** demonstrates the dose-dependent manner of the Notch ligand functionalized microbeads with several qualitative and quantitative assays including myotube inhibition, intracellular Notch receptor activation, real-time RTPCR gene expression analysis and luciferase reporter expression. ***Chapter 5*** illustrates successful T cell commitment in a dose-dependent manner using hematopoietic progenitors, supportive stroma and Notch ligand functionalized beads and immunophenotype analysis. Lastly, ***Chapter 7*** discusses the project insights, conclusions, and future directions. ***Appendix A*** includes a side study we performed on optimization of scaffold properties for hematopoietic differentiation.

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CHAPTER TWO

Background and Significance

2.1 Overview of T Cell Related Immunodeficiencies and Transplantation Strategies

About one in 10000 Americans is born with a maldeveloped thymus, a condition known as Di George's Syndrome (DGS). Early diagnosis and bone marrow or thymic transplantation lead to recovery and minimal discomfort in the quality of life (Bonilla and Geha 2003; Huggins 2006). For those unchecked, however, a lifetime of constant monitoring, prophylactic antibiotics and preventive care rather than corrective care is undergone. In fact, preventive care is the standard for such disorders. Most primary T cell immunodeficiencies such as DGS are disorders of genetic nature and have no effective replacement therapy (Bonilla and Geha 2003; Buckley 2003; Souhami 2002). (**Table 2.1** summarizes some T cell immunodeficiencies with treatment options.)

For conditions such as these, there is a strong need of functional T lymphocytes that can replenish and restore the afflicted's immune system. T cell immunodeficiencies and conditions like cancer rely on transplantations such as bone marrow or adoptive T cell transfer to reconstitute the compromised immune defenses. Such transplantations have displayed a fair amount of success. Indeed, great strides have been made from Till and McCullough's seminal studies in hematopoietic reconstitution in the 1960s (Till and McCollough 1961). For severe combined immunodeficiency patients, for example, bone marrow transplantation success rates have ranged from 50 to 100% (Bertrand et al., 1999; Sinha 2006). Moreover, according to a recent survey conducted by Buckley, 79% of primary immunodeficiency patients transplanted with HLA-identical marrow have lived

(Buckley 2003). Donor type, age at transplantation and severity of condition often dictate the success rate of the procedure; the earlier a condition is diagnosed and treated, the greater the chances of survival (Bonilla and Geha 2003).

Stem cell transplantations have laid much of the groundwork for adoptive T cell transfer, a procedure that has become more common during the last 20 years (Yee 2006). In adoptive transfer, healthy T effector cells are expanded, trained and infused into the patient where they combat and in effect eradicate the preexisting tumor. Effector T cells are particularly well-suited in such *in vivo* applications due to their specificity, long lifespan and ability to be genetically manipulated (June 2007). Adoptive transfer has been beneficial for patients with cytomegalovirus and Epstein-Barr virus-associated post-transplant lymphoproliferative disease resulting in CMV-specific cytotoxic T lymphocyte response restoration and antiviral activity, respectively (Leen et al., 2007). In a recent study by Yee et al, melanoma antigen specific T cell clones were administered with encouraging results in ten patients in a phase 1 trial. T cell clones persisted *in vivo* with little toxicity, homed to tumor site, and removed tumor cells positive for melanoma antigen for almost a two-year period (Yee 2002). The emerging trend, however, is the difficulty in treating immunocompromised patients with malignancies and highly immunosuppressed individuals (Leen et al., 2007). Also, despite, the attractive nature and success of effector T cell transfer, the translation from *in vitro* to *in vivo* clinical trials has been difficult and the procedure is still suboptimal. In the *in vivo*, tumor microenvironment, T cells are short-lived, fail to proliferate and function. Additionally, the process itself can be time, labor and cost intensive and take up to 4-16 weeks, a very long period for individuals with progressive diseases (June 2007; Yee 2005). The need for a robust large-scale production of antigen specific T cells remains (June 2007). Future studies in T cell training, expansion and differentiation have the potential to

significantly benefit cancer and immunodeficiency patients and have a lasting impact on cancer treatment and therapy.

2.2 T CELL DEVELOPMENT AND DIFFERENTIATION

T cell development is comprised of a series of complex interactions that take place both in the bone marrow and thymus and ultimately result in the formation of CD4+ and CD8+ T cells that are capable of recognizing MHC class II or class I molecules, respectively, and informing the immune system of extracellular or intracellular infections. Hematopoiesis, the generation of all blood cells, normally occurs in the bone marrow in the adult mouse (and man). For T cell generation, however, hematopoietic progenitors migrate via the bloodstream in response to chemokines secreted by thymic epithelial cells and through the post-capillary venules into the thymus for T cell development and maturation (Champion 1986; Gill et al., 2003; Goldsby 2003; Wilkinson et al., 1999). It remains unclear whether hematopoietic progenitors have committed to the T cell lineage prior to thymic entry and many reports have demonstrated limited B cell potential of immature thymocytes with *in vivo*, *in vitro* and clonal analysis assays (Balciunaite et al., 2005; Jenkinson et al., 2006; Porritt et al., 2004; Sambandam et al., 2005; Tan et al., 2005; Zediak et al., 2005). Nevertheless, immature thymocytes must undergo a strict development and selection process before they have fully matured. This process can be divided into the double negative stages, β selection, TCR rearrangement, negative and positive selection (Gill et al., 2003; Goldsby 2003). **Figure 2.1** summarizes this process of T cell development delineating the surface marker expression, thymic signals, cell interactions and location of thymocyte development.

Early T lineage progenitors (ETPs) can be identified through their CD44+CD25-CD4-CD8- cell surface marker phenotype. Because these cells are CD4-CD8-, they are

known as double negative (DN). Progression of the differentiation in the DN stage is marked by the surface expression changes of CD44 and CD25 in four stages: DN1 – CD44+CD25-, DN2 – CD44+CD25+, DN3 – CD44-CD25+, DN4 – CD44-CD25-). Notch signaling and interleukin-7 (IL-7) play instrumental roles in driving the differentiation from the DN1 to the DN3 stages, during which the developing thymocytes rearrange the β locus of T cell receptor (TCR) and if successful express TCR α chain and the pre-TCR complex. Selected DN3 thymocytes expand and generate a large and diverse set of CD4+CD8+ thymocytes (known as double positive or DP cells) during β selection that proceed to α chain loci rearrangements and assembly of the αβ TCR-CD3 complex. Next, the developing DP thymocytes interact with the cortical epithelial cells and are positively selected based on their ability to recognize self major histocompatibility complex (MHC) and self peptide. Auto reactive thymocytes are eliminated during negative selection where positively selected thymocytes associate with macrophages and dendritic cells in the medulla and undergo apoptosis. The few cells that survive the selection processes downregulate the coreceptor and develop into CD4+ or CD8+ T cells based on their ability to interact with MHCII and MHCI expressing cells, respectively (Bhandoola and Sambandam 2006; Ciofani and Zuniga-Pflucker 2007; Gill et al., 2003; Goldsby 2003; Guidos 2006). T cells with γδ lineage are also produced in the thymus but a description has been omitted in the interest of length.

2.3 THYMIC MICROENVIRONMENT

Thymocyte development takes place in a complex milieu of supportive cells and extra-cellular matrix (ECM) that are responsible for the proliferation, adhesion, migration, and selection processes thymocytes undergo before reaching maturity (Anderson et al., 2000, Germain 2002; Goldsby 2003). Several of the signals necessary

for thymocyte development and survival which include Notch signaling and MHC-TCR interactions have been characterized through overexpression, gain-of-function and transfection studies (Germain 2002; Goldsby 2003). The thymic microenvironment can be outlined into the following components: molecules (both soluble and cell-bound), extracellular matrix, vasculature, and cells made up of supportive stroma, hematopoietic and non-hematopoietic alike. In a recent paper by Petrie and Zuniga-Pflucker, seven zones have been outlined due to histologic differences and differences in signaling environments. The following description describes the protein and cell-based signals and how they are responsible in dictating the development of thymic progenitors, which is largely coupled to the migration of progenitors from zone to zone. One note to be added is the omnipresent nature of Notch signaling in the thymic microenvironment. Notch signaling is discussed in greater detail below.

As described below, thymic development and differentiation is only possible due to the environmental complexity and interplay of the thymic stroma and developing progenitors. This complex milieu directs the migration, adhesion and proliferation events, characteristic of the T cell differentiation process. In this process, the bone marrow progenitors first must migrate to the perimedullary cortex (PMC) in the thymus through venules, a zone (PMC) rich in homing and adhesion signals such as P-selectin, fibronectin, laminin, VCAM-1, and CCL21. Once the progenitors reach the PMC or Zone 1, DN1 cells expand up to 1000 times with signals from IL-7, Kit ligand, and Hedgehog signaling. Finally, Notch signaling is necessary to prevent non-T cell commitment and takes place due to the increased expression of Delta-like 1 ligand in cortical stroma. In the inner cortex, or Zone 2, upregulation of RAG gene expression in progenitors takes place resulting in TCRγ and TCRδ gene rearrangements. IL-7, which is known to be responsible for survival and proliferation, is present in Zone 2 and also aids

in TCR expression and cell adhesion. Kit ligand is also produced by Zone 2 stroma, aiding in progenitor proliferation. CXCL12 is secreted in the cortex and plays a role in the directional migration of progenitors as they develop. Both VCAM-1 and E-cadherin, expressed on epithelial stroma cells in Zone 2, form the adhesive substrate for migration. Notch ligands are again present on stroma to ensure T cell commitment. In Zone 3 or the outer cortex, DN3 cells migrate to the capsule through the adhesive support and polarity signals of VCAM-1 and CCL25, respectively. Both IL-7 and Notch ligands, also present in Zone 3, are thought to provide the signals necessary for TCRβ locus rearrangement, a key step in committing progenitors to the T cell lineage. Expansion also takes place in Zone 3 but the factors responsible remain unknown. Late DN3 and pre-DP cells undergo completion of TCRβ rearrangements, pre-TCR expression, acquisition of CD4 and CD8 markers and migrate back towards the cortex in the subcapsular zone or Zone 4. Unfortunately, in the area of external stimuli, little besides the large role the preTCR plays in the developmental processes is known. CCL25 and laminin-5 are known to play a role in migration and adhesion, respectively. TNF, present on medullary stromal cells, is thought to play a role in survival, while Notch remains to be a key player in survival and differentiation. CD4CD8 DP cells reside and migrate back towards the cortex in Zone 5. Here, the cells cease proliferation - it is thought through some self-initiated programming and further their maturation. Also, the requirement for stromal matrix-cell contact no longer applies. DP cells still rely on soluble factors and cell-cell contact from stroma to provide the MHC signals, ICAM-1 and IL-7 for positive selection, migration and survival respectively. The outer medulla or Zone 6 is distinguishable from the other zones by its abundance of dendritic cells (DCs), responsible for presenting antigens to developing T cells during negative selection. Medullary epithelial cells, also present in this area, are largely responsible for presenting the self-antigens to the DCs and

thus eliminating auto-reactive T lymphocytes. CD80, CD86, and ICAM-1 also play a part in ensuring effective contact between stroma and T cells. Medullary epithelial cells also express CCL19 and CCL21 ligands which aid the migration of SP T cells toward the medulla. IL-7 and thymic stromal lymphopoietin (TSLP) support SP survival in Zone 6. The final step in SP T cell maturation is a 7-10 day period in Zone 7 or central medulla before they export out of the thymus. MHC-TCR engagement is thought to aid in final development of SP cells and induction of further delineation of T cell types. Additional signals include CD69 expression on stroma which mediate cell retention and lymphotoxin-β receptor necessary for maturation. Edg1, CD69 and G protein-coupled receptors are believed to aid in thymic export but have not completely been confirmed (Anderson et al., 1996; Anderson et al., 2000; Ciofani and Zuniga-Pflucker 2007; Ladi et al., 2006; Petrie and Zuniga-Pflucker 2007; Postlethwaite 1997; Schuurman et al 1997).

2.4 NOTCH SIGNALING

2.4.1 Overview of Notch Signaling

The term Notch signaling was coined in the 1930s when a "notch" was observed in a mutant *Drosophila* wing (Mohr 1919). Although early studies indicated a role in *Drosophila* and neurogenesis, roles in several vertebrate developmental decisions ranging from neuronal differentiation to hematopoietic progenitor expansion were soon observed (Artavanis-Tsakonis et al., 1995; Baron 2003; Chiba 2006; Jarriault et al., 1995). There are four receptors (Notch1-4) that make up the Notch family of receptors. These receptors are highly conserved and are made up of 29-36 epidermal growth factor repeats in the extracellular portion, which are responsible for initiating Notch signaling by binding to Notch ligands. The intracellular portion of Notch transduces the Notch signal

to the nucleus and consists of RAM domain and ankyrin repeats. In mammals, there are 5 ligands - Jagged 1, Jagged 2, Delta 1, Delta 3, and Delta 4. Two main cleavages take place in the transmembrane receptor upon ligand-receptor binding. One cleavage takes place on the extracellular portion and depends on the presence of metalloproteases while the other cleavage occurs in the transmembrane domain generating an intracellular Notch domain (ICN) upon γ-secretase activity. The RAM and ankyrin repeats initiate the binding of the ICN to the helix-loop-helix transciption factor CBF1/RBP-Jk (for mammals), once the ICN has successfully translocated into the nucleus. In the absence of Notch signaling, CBF1/RBP-Jk is bound to corepressors and transcription is blocked. Upon successful Notch signaling, the ICN kicks off the corepressors, and engages proteins such as Mastermind-like proteins, initiating transcription. The complex, however, is eliminated rapidly (Maillard et al., 2005).

2.4.2 Importance in T Cell Development

Notch signaling is well known and perhaps most characterized in determining the fate of developing T cells, evident by the extensive expression of Notch ligands in the thymic microenvironment. These receptors and ligands are tightly regulated in their expression both in the bone marrow and thymus to achieve a unique balance of lymphocyte development (Ciofani and Zuniga-Pflucker 2007; Parreira et al., 2003; Petrie and Zuniga-Pflucker 2007). The expression of all four receptors by the developing thymocytes and the supportive thymic stroma has been previously characterized while Delta ligand (1 and 4) expression has been shown in the thymic stroma (Ciofani and Zuniga-Pflucker 2007; Parreira et al., 2003; Petrie and Zuniga-Pflucker 2007). In situ hybridization studies have shown a high density of Delta 4 ligand, specifically,

throughout the subcapsular zone, cortex and cortical-medullary junction, indicating the importance of the ligand in T cell development (Heinzel et al., 2007).

Gain-of-function, overexpression, and loss of function studies have been instrumental in understanding the necessity of Notch signaling in thymocyte development and survival. In studies with constitutively active forms of the Notch ligand in HSCs, B cell development was inhibited entirely and extrathymic T cell generation was observed (Pui et al., 1999). Defects of Notch1 gene also led to deficiencies in T cell development and an increase of B cell development in the thymus (Radtke et al., 1999). Similar results were observed in CSL based studies. CSL is known for controlling the signaling of Notch receptors. When bone marrow progenitors that were deficient in CSL were placed in the thymus, T cells failed to form, leading to development of B cells (Han et al., 2002). Additional gain-of-function studies demonstrated ectopic T cell development in the bone marrow microenvironment upon Notch1-IC or Delta 4 expression in progenitors (Dorsch et al., 2002; Yan et al., 2001). These studies combined with what is known on the thymic microenvironment reveal the vital role Notch ligands play in T cell fate decisions.

2.5 *EX VIVO* T CELL DIFFERENTIATION METHODS

2.5.1 Fetal and reaggregate thymic organ cultures

Immense progress has been made in the area of *ex vivo* T cell differentiation in the last forty years, leading to a wealth of insights in T cell development and lymphopoiesis. Early studies relied on fetal thymic organ culture (FTOCs) system for T cell development, largely established by Owen and Jenkinson. This was mostly due to the widely held belief that the thymus and its architecture particularly, were indispensable in T cell development. In FTOCs, the thymic lobes are stripped of thymocytes with

chemical treatment and used to provide their unique architecture and signaling to thymic progenitors (de Pooter and Zuniga-Pflucker 2007; Lehar and Bevan 2002; Zuniga-Pflucker 2004). FTOCs were the established means of studying T cell development and elucidated the underpinnings of the field. For example, Yeoman and colleagues demonstrated the ability to generate CD4+ and CD8+ SP T cells from human bone marrow and umbilical cord blood cells in FTOCs with varying differentiation capabilities observed between progenitor populations (Yeoman et al., 1993). Additional FTOC based studies supplied important detail on mechanisms of T cell development including differentiation abilities of various progenitor populations, importance of cell cycle and effect of cytokines, to name a few (Plum et al., 1994; Res et al., 1996; Toki et al., 1991; Weekx et al., 2000). Reaggregate thymic organ cultures (RTOCs), which consists of disaggregated stroma that have subsequently been recombined, were also beneficial for such studies (Anderson et al., 1993). FTOC and RTOC based cultures though seminal in their contributions to T cell development and vital even today for their ability to provide adequate environments for T cell selection, suffer from low cell differentiation efficiencies, varied results, complicated setup, and difficulties in isolating distinct signals (de Pooter and Zuniga-Pflucker 2007; Zuniga-Pflucker 2004).

2.5.2 Recent methods: thymic stroma, cytokine cocktails, stromal cell lines

Developments in scaffold technology led to the application of thymic stroma to tantalum based matrices, readily used in bone repair and the efficient generation of human T cells with up to 70% CD3+ T cells after 14 days of stroma-human CD34+ cell coculture (Poznansky et al., 2000). Further studies illustrated successful positive and negative selection in these matrices with E1 and OVAp peptide addition, respectively (Marshall et al., 2003). Time needed to establish thymic structure in 3D cultures was still

a potential disadvantage of the system. Another method readily used requires the addition of exogenous cytokines such as stem cell factor, IL-7, flt-3 ligand, IL-6, IL-7, IL-3, and thrombopoietin and immobilization of Delta ligands at various concentrations. This method is especially attractive due to its quantitative and cell-free nature and ability to be scaled up. Using this method, Bernstein and colleagues have elegantly demonstrated the dose-dependent effects of immobilized Notch ligand Delta1 on cord blood cell derived T cell differentiation and thymus repopulation (Delaney et al., 2005; Ohishi et al., 2002). Higher ligand densities resulted in an increased CD34-CD7+ differentiation efficiency and interestingly enough lower SCID reconstitution ability (Delaney et al., 2005). Thy1+CD25+ expression from lin-sca-1+c-kit+ murine bone marrow hematopoitic precursors was also shown upon exposure to increasing amounts of DLL1 with higher densities inhibiting the development of B cells (Dallas et al., 2005). One disadvantage of the method, however, remains the expense associated with supplementary cytokines. One alternative to exogenous cytokines is a supportive cell line that in turn secretes relevant cytokines. Use of cytokine secreting stromal cell lines has in fact been extensively utilized in lymphoid differentiation (Cho et al., 1999; Gutierrez-Ramos and Palacios 1992; Jaleco et al., 2001; Landreth and Dorschkind 1988; Nakano et al., 1996). Cell lines such as RP.0.10, S17, and OP9 generate and secrete lymphoid cytokines such as IL-7 and SCF (for OP9 for example) that aid in proliferation and survival (Cho et al., 1999; Gutierrez-Ramos and Palacios 1992; Jaleco et al., 2001; Landreth and Dorschkind 1988; Nakano et al., 1996). Furthermore, OP9 cells, derived from the OP/OP mice, fail to produce macrophage colony-stimulating factor, a factor known to drive myeloid differentiation, thus further aiding in lymphopoiesis (Zuniga-Pflucker 2004). Despite the success in generating hematopoietic cell lineages including

B cells, erythrocytes, and NK cells, the generation of T cells using these stromal cell lines was unsuccessful (Zuniga-Pflucker 2004).

2.5.3 Current method: OP9-DL1 cell line and spinoffs

As indicated above, Notch signaling is both omnipresent and indispensable in T cell development. DLL1, for example, is particularly distributed in high densities in the thymic cortex and cortical-medullary junction (Ciofani and Zuniga Pflucker, 2007). These findings led to one of the greatest tools in T cell differentiation research today: the creation of a DLL1 expressing OP9 cell line (de Pooter and Zuniga-Pflucker 2007; Lehar and Bevan 2002; Pear 2005; Rolink et al., 2006; Zuniga-Pflucker 2004). The emergence of a Notch expressing cell line pushed the T cell differentiation field forward with a flood of seminal studies following. The *in vitro* system is based on the OP9 stromal cell line utilized for B cell differentiation (described above) but takes advantage of Notch signaling to drive the differentiation toward the T cell lineage.

Zuniga-Pflucker and colleagues, the makers of the OP9-DL1 cell line, have contributed significantly and continue to contribute to the field today. Early studies with the OP-DL1 cell line led to the induction of T cell differentiation in murine fetal liver cells, embryonic stem cells and bone marrow derived HSCs as indicated by CD4CD8 marker expression and T cell specific gene transcripts and/or TCR rearrangements. Functional SP CD8+ TCRαβ T cells can be observed using the *in vitro* system after 22 days of culture. The resulting T cell population does not, however, include any CD4+ T helper cells unless transported to a FTOC for the latter half of the differentiation process. The absence of MHC II+ cells in the *in vitro* system is thought to be responsible (de Pooter and Zuniga-Pflucker 2007; Schmitt et al., 2002; Schmitt et al., 2004).

Significant developments were made in differentiation from human sources, as well. A 2004 study indicated the generation of DP αβ T cells from a CD34+ bone marrow source within 35 days of coculture (De Smedt et al., 2004). La Motte-Mohs and colleagues demonstrated the generation of pre-T CD7+CD1a+ cells and DP αβ T cells within 24 days of coculture with OP9-DL1 and CD34+CD38- human cord blood derived HSCs (La Motte-Mohs et al., 2005). More recently, Zhao et al generated p53 or ESO tumor antigen specific T cells, the holy grail of T cell research, using the OP9-DL1 coculture system. Human cord blood derived CD133+/CD34+ cells were transfected with TCR expressing vectors with reactivity against tumor antigens p53 or NY-ESO-1. GMCSF and IL-2 secretion was observed upon T cell incubation with peptide-pulsed antigen presenting cells (Zhao et al., 2007). Also, Lefort and colleagues demonstrated that a short 4 day exposure to immobilized DLL4 ligand was sufficient enough to induce CD7+cytCD3ε+ phenotype, relevant T cell transcription factors and TCR rearrangements in CD34+ cord blood cells. Further incubation with DLL4 expressing OP9 cell line or FTOC culture initiated DP status in developing T cell progenitors (Lefort et al., 2006).

The introduction of Notch ligands into supportive cell lines such as OP9 has stimulated similar approaches of Notch signaling and stroma for T cell development. Hozumi and colleagues first demonstrated the importance of intracellular Notch1 coupled with OP9 and IL-7 to T lymphopoiesis in their 2003 study where intracellular Notch 1, 2 or 3 were transfected into hematopoietic progenitors. Results indicated DP Thy1.2+ cells for all three Notch receptors (Hozumi et al., 2003). One year later, the same group demonstrated the differentiation of cKit+ fetal liver cells into Thy1.2+ T cell progenitors with TCR arrangements upon incubation with OP9-DLL4 stromal cells (Hozumi et al., 2004). Lehar and colleagues used OP9 transfected Jagged 1 and OP9 transfected Delta 1 cell lines to demonstrate the importance of Delta 1 and not Jagged 1 in the induction of

maturation and expansion of T cell precursors (Lehar and Bevan 2005). Finally Jaleco et al successfully generated DP T cells using DL1 transfected S17 stromal cell line (Jaleco et al., 2001).

2.5.4 Limitations of OP9-DL1 system

The OP9-DL1 based system of T cell differentiation has eliminated the inconvenience associated with FTOCs and stroma based systems, the previous standard in *in vitro* T cell differentiation. The far-reaching impact of the OP9-DL1 system lies in its ease, efficiency, reproducibility and ability to be manipulated (Zuniga-Pflucker 2004). However, some drawbacks inherent in this system have prevented the generation of functional CD4 cells and reduced the efficiency of the system for the eventual high throughput generation of T cells. First, the generation of functional CD4 SP cells has yet to be demonstrated using Notch based systems. OP9 cells fail to express the MHC II molecule and provide the microenvironment necessary for selection, both of which are necessary for CD4 T cell differentiation (de Pooter and Zuniga-Pflucker 2007; Zuniga-Pflucker 2004). Another fundamental limitation is the dependence of OP9 based systems on transfected cells for Notch signaling. The transfection of stroma cells for different Notch ligands can become cumbersome and interfere with normal genetic expression of the OP9 cell (Lehar and Bevan 2005). In addition, the inherent design of the current coculture system makes large-scale T cell generation difficult, specifically for 3D studies in biomimetic environments. Also, a systematic quantification of ligand amount is difficult in OP9 based studies due to cell dependent ligand presentation.

2.5.5 Motivation for DLL4 based T cell differentiation

The high density of the Notch ligand in the thymic microenvironment, the gain-of-function studies, and *in vitro* studies demonstrating successful T cell specification upon DLL4 exposure all indicate the significance of the ligand in thymocyte development. Despite the insightful studies provided by Hozumi et al and Lefort et al, studies utilizing Notch ligand DLL4 in ex vivo T cell development have been limited (Hozumi et al., 2004; Lefort et al., 2006). Further studies elucidating the quantitative and temporal effects of Notch ligand DLL4 would provide immense insight into the density dependence of Notch signaling in lymphopoiesis, specifically the T cell commitment and maturation as implicated in recent papers by Dallas et al, Ciofani et al and Lehar et al (Ciofani et al., 2005; Dallas et al., 2005; Lehar and Bevan 2005). These studies would also help understand the separate roles, if any, of the Notch ligands DLL1 and DLL4 in T cell development.

2.6 BEADS IN CELL STUDIES

2.6.1 Overview of beads

Magnetic antibody-coated beads have been widely used for immunoseparation applications for almost 20 years (Egeland et al., 1991; Swann et al., 1992). More recently, however, magnetic beads are being applied to a more diverse set of cell based problems, due to their obvious separation applications, commercial availability, ability to study cellular processes on a micro scale, and ability to be molecularly defined and optimized. Two studies, for example, have exploited the magnetic nature of the beads to characterize the localization and trafficking of EGF based signals and vesicles with the use of electron microscopy, respectively (Friedlander et al., 2005; Li et al., 2005).

Glycine receptor diffusion has also been tracked microscopically using 0.5-μm beads, though not magnetic, coated with anti-myc antibodies (Craig and Lichtman 2001). Another study by Fass and Odde studied force dependent elongation and retraction in embryonic chick forebrain neurons using anti-β1 integrin antibody coated magnetic beads (Fass and Odde 2003). The most extensive use of bead, magnetic or not, however, has been in T cell activation and proliferation (Ito et al., 2003; June 2007; Kim et al., 2004; Maus et al., 2002; Maus et al., 2003; Trickett et al., 2002). In these studies, beads are decorated with T cell relevant costimulatory molecules for the expansion of patient derived T cell populations and the generation of antigen specific CD4 and CD8 populations. Levine and colleagues first demonstrated up to $10^9 – 10^{11}$ fold expansion in CD4+ T cells with antiCD3 and antiCD28 antibody coated 4.5μm magnetic Dynalbeads for a more than 30 day culture (Levine et al., 1997). Trickett et al further characterized magnetic Dynalbeads for CD4+ expansion and cytokine production, albeit with a different type of magnetic bead, examining culture conditions such as presence of serum, number of beads and stable vs. dynamic culture (Trickett et al., 2002). In another study, indirect and direct conjugation schemes of HLA tetramer to magnetic beads were evalulated based on amount of HLA monomer/tetramer loading and ability to activate CD4+ T cells. Beads using an indirect binding scheme where the tetramer was not directly bound to the bead produced the optimal results. Maus and colleagues' anti-CD3 and antiCD28 beads are in fact in clinical trials for adoptive immunotherapy applications, perhaps the greatest indicator of how much progress has been made with such beads (Maus et al., 2003).

2.6.2 Motivation for beads as a method for protein presentation

Historically, there have been several methods of displaying proteins on surfaces such as liposomes and immobilization onto polystyrene (Engelhard et al., 1978; Kane et al., 1989). Often times, proteins cannot be recognized by its associated receptor in the soluble form, especially when such proteins are normally presented on the surface of a cell, thus necessitating such methods of protein immobilization. For example, soluble monomeric or dimeric Delta-1 fusion ligands failed to activate Notch receptors in C2C12 and U20S cells as indicated by differentiation and HES1 transactivation studies, respectively (Varnum-Finney et al., 2000).

Beads offer several benefits in protein display. For applications such as ours, where scaling up the production of T cells is a goal, such bead systems are ideal. The ability to define and optimize a system reproducibly in a more cost-effective manner, free of safety concerns presented in cell systems, makes the bead approach an attractive option. Beads may in fact be more ideal than methods such as plate immobilized protein due to the ability to reach several cell layers and provide greater surface area. In a study by Ito and colleagues, bead conjugated antibodies resulted in higher levels of T ell activation as compared to plate immobilized antibodies (Ito et al., 2003).

Table 2.1 Table of Summary of T Cell Related Immunodeficiencies

Table 2.1 Summary of T Cell Related Immunodeficiencies

Name of Immunodeficiency	Description	Prevalence	Treatment options	References
Di George's syndrome	Abnormal development of thymus	(US) 1:70000 people; 1:10000 births, immune deficiency recovers with time	*Severe cases:* bone marrow transplantation *Moderate T cell function:* preventive antibiotics	*, **, ***, ****
Ataxia-telangiectasia	Defective double strand break enzyme; TCR genes affected; radiation sensitivity	(World) 1:100000 births	No specific treatment available, preventive antibiotics, aid with infections	*, **, ***, ****
T cell antigen receptor complex deficiency	Impaired T cell antigen receptor signaling due to gene mutation	Not found		*, ***
Major histocompatibility complex class II deficiency	Absence of MHC class II molecules on APCs resulting in incomplete CD4+ T cell development	Not found		*
Nude syndrome	*whn* gene mutation resulting in impaired epithelial thymic development	2 people reported till now	Bone marrow transplantation	*, IDR file
Chronic mucocutaneous candidiasis	Disorders prone to frequent skin, mucous membrane, nail infections with *candida* yeast	Not found	Antifungal agents, immunologic therapies, and combination therapy.	**, ******
Wiskott-Aldrich syndrome	Genetic defect in Wiskott-Aldrich syndrome protein resulting in impaired cytoskeletal actin function and T cell activation	(US and world) 4:1 million male births	Bone marrow transplantation in early childhood, infection treatment	**, ***, *******
Severe combined immunodeficiency	Disorders resulting from genetic mutations; differentiation, proliferation of T cells inhibited	(US) 1:50-75000 births	Bone marrow transplantation, preventive antibiotics, gene therapy for some types	*, **, ***, ********

NOTE: Descriptions have been limited to T cell related effects

* (Kasper et al., 2005)

** (Souhami, R, 2002)

*** (Bonilla, F and R.S. Geha, 2003)

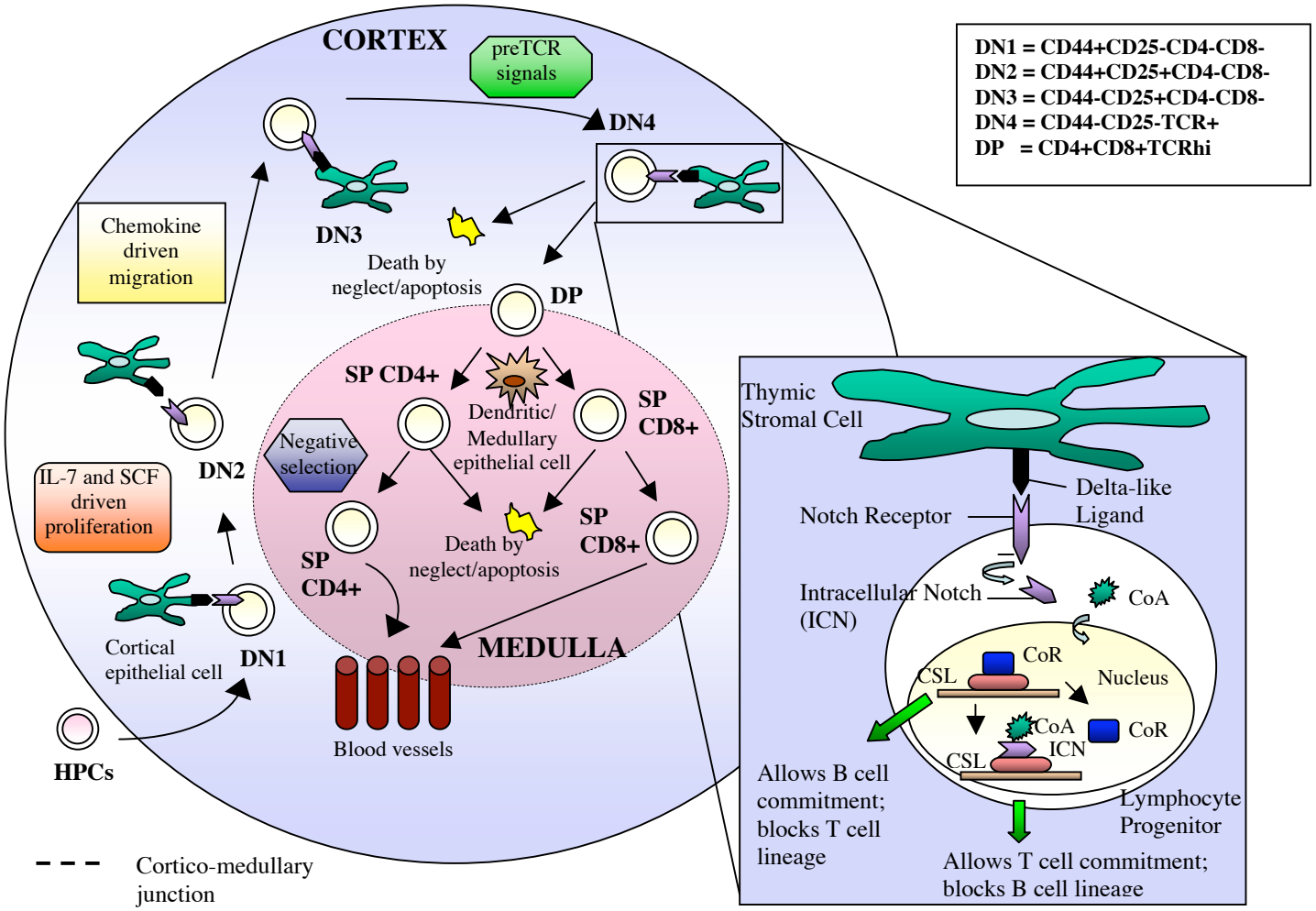
**** (Huggins, R.H., 2006)

***** (IDR file reference)

****** (Roberts, D.T., 2006)

******* (Dibbern, D.A., 2005)

Figure 2.1 T cell development in the thymic microenvironment. Notch signals, IL-7, SCF, chemokine gradients and preTCR signals all drive the development of DN population to a DP developing thymocyte. In the medulla, DP cells undergo negative selection through interactions with dendritic and medullary epithelial cells and emigrate out of the thymus near the cortical medullary junction. Thymocytes undergo death by neglect and apoptosis at both the DN=> DP transition and during negative selection.



Modified from Zuniga-Pflucker, J.C. 2004. T cell development made simple. Nature Reviews Immunology *4*, 67-72.

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CHAPTER THREE

Development and Characterization of Notch Ligand Coated Microbeads

3.1 INTRODUCTION

Current efforts in ex vivo T cell differentiation have eliminated the inconvenience associated with fetal thymic organ cultures (FTOCs) and stroma based systems, the previous standard *in vitro* T cell differentiation (Han et al., 2000; Kennedy et al., 2003; Parreira et al., 2003; Plum et al., 1994; Poznansky et al., 2000; Res et al., 1996; Weekx et al., 2000; Yeoman et al., 1993; Zuniga-Pflucker and Schmitt 2005). These studies have reported a need for Notch signaling in T cell differentiation, leading to novel ways of introducing Notch ligands into *in vitro* culture. Efforts have relied largely on Notch ligand transfected stroma to provide signals critical to T cell development and have successfully demonstrated effective T cell progenitor generation (Hozumi et al., 2003; Hozumi et al., 2004; La Motte-Mohs et al., 2005; Lehar et al., 2002; Schmitt et al., 2002; Schmitt et al., 2004; Zhao et al., 2007). The transfection of stroma cells for different Notch ligands, however, can become cumbersome and interfere with normal genetic expression of the OP9 stromal cell (Lehar et al., 2005). Recent studies have also utilized plate adsorbed Notch ligand, supplying the signal in a quantitative and cell-free manner (Beckstead et al., 2006; Dallas et al., 2005; Delaney et al., 2005; Lefort et al., 2006; Ohishi et al., 2002). This method presents significant benefits for scaling up *ex vivo* T cell generation for possible therapeutic applications. Nevertheless, plate adsorption for ligand presentation can become expensive and unstable with extended cell culture (due to extracellular matrix secretion from cells).

T cell activation and cell separation applications have frequently utilized functionalized microbeads for cell targeting and providing specific signals to these cell targets (Connors et al., 2005; Curtsinger et al., 1997; Ito et al., 2003; Levine et al., 1997; Maus et al., 2002; Maus et al., 2003; Patel et al., 1995; Pilling et al., 1989; Sarda et al., 2004; Trickett et al., 2002). Microbeads offer several benefits in applications such as these, including ease in handling and fabrication, low cost, amenable to scaling up and effective protein (antibody or ligand) presentation (Curtsinger et al., 1997; Maus et al., 2003). For example, to activate and expand CD4+ T cells for clinical applications, protocols require immobilized anti-CD3 and anti-CD28 antibodies to effectively recognize and bind to their respective antigens upon the T lymphocytes (Levine et al., 1996). Studies have functionalized these antibodies onto microbeads and demonstrated up to 10^{11}-fold expansion of CD4+ T cells upon incubation with microbeads in the absence of supportive feeder layers (Levine et al., 1997; Maus et al., 2002; Trickett et al., 2002). A recent study has additionally demonstrated increased proliferation and tumor-specific cytokine secretion with CD4+ cells when comparing bead-activated and plate-activated cells (Ito et al., 2003). The increased surface area may additionally improve the efficacy of such an artificial cell construct. Long-term stability of functionalized beads has also been shown up to 60 days in long-term T cell proliferation studies (Levine et al., 1997).

In this study, we report a synthetic Notch signaling system using ligand-functionalized magnetic microbeads (artificial stromal cells) that can ultimately be used to evaluate how Notch ligands, specifically DLL4, presented through a biomaterial surface affect T cell differentiation and to eventually develop a high throughput strategy to engineer T cells from hematopoietic progenitor populations. We functionalized streptavidin-coated microbeads with DLL4 using biotin-streptavidin chemistry and

antigen-antibody coupling and demonstrated their functionality using flow cytometry and confocal microscopy. Further, we optimized the functionalization process in terms of key parameters such as protein and bead concentration and incubation time. Additional stability studies were also carried out where effect of buffer and serum on bead functionalization over time were evaluated. Poor results in stability testing prompted us to switch to another bead type, which was in turn characterized in terms of protein concentration effects, stability testing and visualization through microscopy. (Differences in characterization results between the two beads stemmed from the presence of a DNA linker on the first type of bead.) Finally, we demonstrated low cellular toxicity with beads using MTT viability test for C2C12 myoblasts and R1 embryonic stem cells.

These results demonstrate a robust, non-toxic functionalized bead system that can be used for signaling studies in a variety of biological applications. Such a bead-based artificial signaling system could allow us to quantitatively study the effects of ligand density and signaling duration thereby providing further insights into the individual roles of the Notch ligands in T cell differentiation and ultimately aid in the development of efficient technologies for the production of T cells for therapeutic applications.

3.2 Materials and Methods

3.2.1 Biotinylated polyhistidine antibody functionalization and optimization

Biotinylated antibodies specific for a histidine tag on recombinant DLL4 were coated onto streptavidin coated superparamagnetic polystyrene microbeads and optimized in terms of the following conditions: quantity of antibody, incubation time and incubation concentration. Biotin Binder Kit microbeads (Dynalbiotech, Brown Deer, WI) were washed with 0.1% bovine serum albumin (Sigma, St. Louis, MO) in PBS and incubated

for 30 min at room temperature on a rotator with 10, 20, 50, 100, 200, 400, 600, 800, 1000, 1500, 2000, and 2100 ng of biotinylated anti-6x HIS tag antibody (R&D Systems, Minneapolis, MN) per 10^7 beads at a concentration of 80000000 beads/ml. After incubation, beads were washed three times and stained as described below. Incubation time and concentration studies were performed using the optimized biotinylated anti-6x HIS tag antibody concentration (100 ng/10^7 beads) for 30 min, 60 min, and 90 min and with concentrations of 80000000 beads/ml, 800000 beads/ml, and 8000 beads/ml. Beads were then washed and stained as described below.

3.2.2 DLL4 functionalization and optimization

Recombinant DLL4 (R&D Systems, Minneapolis, MN) was functionalized onto anti-6x HIS tag antibody coated microbeads as described above and was first stained to verify effective DLL4 functionalization. In these preliminary studies, Biotin Binder Kit microbeads (Dynalbiotech, Brown Deer, WI) were washed and incubated with anti-6x HIS tag antibody (R&D Systems, Minneapolis, MN) at 1 μg/ml for 30 min at room temperature. After incubation, beads were again washed and further incubated with the HIS-tagged DLL4 protein (R&D Systems, Minneapolis, MN) at 2-4 μg/ml for 30 min at room temperature. Beads were washed and stained as described below. Both uncoated and anti-6x HIS tag antibody beads served as controls. Optimization studies were then performed which included the following conditions: quantity of DLL4, incubation time and incubation concentration. DLL4 binds to the anti-6x HIS tag antibody beads through its histidine tag at the carboxy-terminus. Biotin Binder Kit microbeads were first washed and coated with 100 ng of biotinylated anti-6x HIS tag antibody/10^7 beads for 30 min at a concentration of 80000000 beads/ml. After adequate washing, microbeads were then incubated for 30 min at room temperature on a rotor with 10, 20, 50, 100, 200, 500, 1000,

and 2000 ng of DLL4 per 10^7 beads at a concentration of 80000000 beads/ml. After incubation, beads were washed three times and stained as described below. Incubation time and concentration studies were performed using the optimized DLL4 concentration (1000 ng/10^7 beads) for 30 min, 60 min, and 90 min and with concentrations of 80000000 beads/ml, 800000 beads/ml, and 8000 beads/ml. Beads were then washed and stained as described below. Additionally, DLL4 characterization studies were conducted for streptavidin-coated magnetic microbeads without DNA linkers. Proactive® streptavidin coated magnetic beads (Bangs Labs, Fishers, IN) that were 5.91 μm in diameter were coated with biotinylated anti-6x HIS antibody based on manufacturer's recommended protocol. Beads are provided with a lot specific biotin-FITC saturation capacity that was converted based on molecular weight to a saturation capacity for biotinylated anti-6x HIS antibody. For DLL4 titration studies, DLL4 concentrations of 20, 40, 100, 200, 400, 600, 1000, 2000, and 4000 ng of DLL4 per million beads were coated onto biotinylated anti-6x HIS antibody coated beads as per manufacturer's instructions. Beads were washed and stained as described below.

3.2.3 Bead stability testing

To test the integrity of the DLL4 coating on the microbead surface, functionalized microbeads were incubated for up to a week at 4ºC in 0.1% BSA in PBS at a concentration of 400000000 beads/ml and stained for DLL4 at the time of fabrication and 6 days afterward in the manner described below. Additional studies were performed to characterize the integrity of the functionalized microbeads in various buffers. Functionalized microbeads were added to 48 well plates and cultured for 1 h at both 4ºC and 37ºC (in a humidified incubator supplemented with 5% CO_2) in 0.1% BSA in PBS, serum-free C2C12 differentiation medium and serum supplemented C2C12 medium.

Several signaling and differentiation studies are performed with C2C12 myoblasts and thus the media listed are used frequently. Serum-free medium was prepared as described in studies by Chan et al (Chan, X. C. Y. et al., 2007). Medium consisted of 1:1 ratio of Dulbecco's modified eagle medium and F12 nutrient mixture (Invitrogen, Carlsbad, CA), 1mM sodium pyruvate (Invitrogen, Carlsbad, CA), 2.5 μg/ml bovine insulin (Sigma-Aldrich, St. Louis, MO), and antibiotics. Serum supplemented medium consisted of Dulbecco's modified eagle medium, 10% fetal bovine serum (Hyclone, Logan, UT) and antibiotics. DLL4 staining (as described below) was performed for all conditions after incubations. Anti-6x HIS antibody functionalized beads were used as controls. Beads were stained with 100ng anti-6x HIS antibody per 10 million beads and 1000 ng DLL4 per 10 million beads. Proactive® streptavidin coated magnetic beads were also tested for integrity of DLL4 coating. Beads were stained with calculated anti-6x HIS antibody concentration described above and 700 ng of DLL4 per million beads and incubated for 1 hr, 1 day and 1 week timepoints in PBS at 4ºC and 37ºC and C2 serum supplemented and serum-free media with anti-6x HIS antibody coated beads as negative controls. Staining was performed for all conditions after incubations.

3.2.4 Immunofluorescence and flow cytometry analysis

Conjugation of protein immobilization onto both Dynalbiotech and Bangs Labs microbeads was confirmed by visualization through fluorescently labeled antibody staining for relevant protein and flow cytometry analysis. Following anti-6x HIS tag antibody and DLL4 binding, microbeads were blocked for ≥2 hr at 4ºC with 3% bovine serum albumin (Sigma-Aldrich, St. Louis, MO) in PBS. Microbeads were then stained with streptavidin-PE (BDBiosciences, San Jose, CA) at 0.25 μg per 10^6 beads in 1% bovine serum albumin in PBS with 0.05% sodium azide (Sigma-Aldrich, St. Louis, MO)

for 30 min at 4ºC while rotating to detect antibody immobilization. After staining, beads were washed three times, resuspended in 250 μl of staining buffer and analyzed using FACSCalibur (Becton Dickinson, San Diego, CA) and CellQuest 3.1 software (BD Biosciences, San Jose, CA). Uncoated microbeads served as controls for antibody staining while anti-6x HIS tag antibody beads served as controls for DLL4 staining. Fluorescence from microbeads was subtracted from samples to adjust for background. Staining for antibody optimization studies was also performed using FITC conjugated goat anti-mouse IgG (Calbiochem, San Diego, CA) at the concentration for streptavidin-PE given above. Results were similar to streptavidin-PE results shown below (data not shown). For DLL4 binding, blocked microbeads were stained with anti-mouse DLL4 antibody (R&D Systems, Minneapolis, MN) at 2 μg per 10^6 beads in 1% bovine serum albumin in PBS with 0.05% sodium azide (Sigma-Aldrich, St. Louis, MO) for 30 min at 4ºC while rotating. Beads were then washed three times and stained for an additional 30 minutes at 4ºC with 0.25 μg of FITC anti-rat IgG (eBiosciences, San Diego, CA) per 10^6 beads while rotating. After incubation, beads were washed, resuspended and analyzed as described above. Immobilized DLL4 molecules on bead surfaces were quantified using the anti-rat IgG Quantum Simply Cellular Kit (Bangs labs, Fishers, IN) beads. Anti-rat IgG Quantum Simply Cellular (QSC) Kit beads consist of 5 bead types with increasing amounts of known goat ant-rat IgG on their surfaces, from which a linear curve can be produced and mean fluorescence can be converted to molecules of equivalent soluble fluorochrome (MESF) from the measuring bead population assessed. QSC beads were stained similarly to the DLL4 immobilized beads.

3.2.5 Confocal microscopy

Functionalized microbeads were also examined using confocal microscopy to ensure intact DLL4 coating around the entire surface using a similar staining scheme as described above. Instead of the FITC anti-rat IgG, APC anti-rat IgG (Invitrogen, Carlsbad, CA) was used at the concentration given above. Beads were washed with ddH_2O three times after staining to prevent crystal formations and resuspended in 100 μl of ddH_2O. Bead-water suspensions were added to glass slides and dried over night in the dark. Beads were then mounted with immersion oil (Sigma Aldrich, St. Louis, MO), covered with coverslips, sealed with clear nail polish and imaged using a Leica SP2 AOBS Confocal Microscope (Leica Microsystems, Bannockburn, IL). Anti-6x HIS antibody-coated beads were used as controls. Samples were imaged using reflected light, transmitted light and fluorescence with an excitation of 633 nm with a He-Ne laser. Similar procedure was performed for Proactive® streptavidin coated magnetic beads with FITC anti-rat IgG staining.

3.2.6 Quantification of DLL4 conjugation using ELISA

The amount of DLL4 adsorbed onto the Biotin Binder Kit microbeads was quantified using an ELISA assay. Briefly, beads were coated as described above. Washes (in 0.1% w/v bovine serum albumin in PBS) from the bead conjugation process were stored at -20°C for ELISA analysis. Standard wells of 96 well EIA/RIA ELISA plates (Corning, Corning, NY) were incubated with the wash samples and standards over night at 4°C. Plates were then washed 4x with 0.5% (v/v) Tween 20 in PBS (PBT) and blocked with 1% (w/v) bovine serum albumin in PBS for 1.5 h at 37°C. After blocking, plates were washed 4x with PBT and incubated for 1.5 h with rat anti-mouse DLL4

IgG2a (R&D systems, Minneapolis, MN) at a concentration of 1 μg/ml at room temperature followed by extensive washing. Finally, HRP conjugated goat anti-rat IgG (1:5000, eBioscience, San Diego, CA) was added to the wells. After 1.5 h incubation, wells were washed with PBST and developed using tetramethylbenzidin (TMB, R&D systems, Minneapolis, MN). Absorbance values were read at 450 nm using the Opsys MR Microplate reader (Dynex. Technologies Inc., Chantilly, VA) and Revelation QuickLink software (Thermo Labsystems, Chantilly, VA). All samples and standards were analyzed in triplicate. Amount of DLL4 conjugated to microbeads were obtained by subtracting the measured amount of DLL4 in the washes from the original amount of DLL4 added to the beads.

3.2.7 MTT assay

To assess the cytotoxicity effects of the Biotin Binder Kit magnetic beads with or without functionalized DLL4, a MTT assay was performed. R1 or C2C12 cells were cultured with both functionalized and uncoated Biotin Binder Kit beads for 3 days, 5 days and 7 days at bead to cell ratios of 0.1:1, 1:1 and 5:1. Briefly, undifferentiated R1 embryonic stem cells (a gift from A. Nagy, Mount Sinai Hospital, Ontario, Canada) were expanded on LIF (leukemia inhibiting factor)-producing irradiation-inactivated embryonic fibroblast cells (STO cells, Shan Maika, UT Austin) for 10 days. R1 embryonic stem cells were seeded at 10000 cells/well in complete DMEM medium (Invitrogen, Carlsbad, CA) containing 20% fetal bovine serum (Hyclone, Logan, UT), 2 mM L-glutamine, 1 mM sodium pyruvate, 0.1 mM non- essential amino acids, 5 x 10^{-5} M 2-mercaptoethanol, penicillin G (100 U/mL), and streptomycin (10 μg/mL) (all from Invitrogen, Carlsbad, CA) while C2C12 myoblasts were seeded at 20000 cells/well in 96 well plates one day prior to bead addition in C2C12 maintenance medium, consisting of

DMEM (Invitrogen, Carlsbad, CA), 10% characterized fetal bovine serum (Hyclone, Logan, UT) and antibiotics. Cells were counted using a hemocytometer the day of bead addition to confirm accurate cell number for bead to cell ratios. Additional studies used uncoated beads only at bead to cell ratios of 0.1:1, 1:1, 10:1, 20:1 and 40:1 with the timepoints given above. For each timepoint, plates were removed from incubator and medium was aspirated. Fresh medium (100 μl) along with 10 μl of 5 mg/ml MTT (Sigma Aldrich, St. Louis, MO) in Hank's Balanced Salt Solution (Invitrogen, Carlsbad, CA) was added to each well. A medium only condition was included as background. Plates were incubated at 5% CO_2 in 37°C for 4 hours. After incubation, 100 μl of MTT solubilization solution (10% Triton X-100, 0.1 N HCl in anhydrous isopropanol) was added and pipetted several times to dissolve purple formazan crystals. The supernatant was removed from each well and centrifuged at 300 g for 5 minutes. Without disturbing the pellet, 100 μl of each sample were added to new 96 well plates and read at a wavelength of 570 nm using the plate reader. All values were exported and analyzed in Microsoft EXCEL. Medium only absorbance values were subtracted from all samples. Cells without beads were used as 100% viability and samples were converted to percentages and plotted. DLL4 functionalized beads were coated with 1 μg/ml of anti-6x HIS tag antibody and 1 μg/ml of DLL4 with optimized incubation volumes and times.

3.2.8 Statistical analysis

All experiments were performed in at least an n=3. Standard error was calculated. Analysis of statistical significance was performed using a one tailed Student's *t*-test. Significance was determined using a 95% probability value ($p<0.05$). All experiments were repeated twice. Representative data is shown.

3.3 RESULTS

In past studies, Notch ligands such as DLL1 and DLL4 have been presented as retrovirally transfected cell-surface ligands in lymphoid differentiation applications, making ligand quantification and scale-up difficult. To address this quantitative hurdle and to develop a biomaterial-based artificial Notch-signaling system, our eventual goal is to evaluate whether the Notch ligand DLL4, immobilized on the surface of magnetic microbeads can function in T cell differentiation similar to when presented on the surface of stromal cells. Here, DLL4 was attached to super paramagnetic microspheres using conventional biotin-streptavidin chemistry and antibody-antigen binding. Our goal in the functionalization process was to (a) present the ligand in an efficient manner and (b) preserve the conformational structure of the ligand during the process. We evaluated this design for microspheres with and without a DNA linker (with the DNA linker providing separation capacity). **Figure 3.1** shows the schematic structure of the DLL4 functionalized microbeads (a) with a DNA linker (b) without a DNA linker.

3.3.1 Optimal conditions for biotinylated polyhistidine antibody functionalization to microbeads

Biotin Binder Kit microbeads are 4.5 μm streptavidin coated superparamagnetic polystyrene beads that are readily used for cell-bead separation applications. The streptavidin coating is coupled to the polystyrene bead via a DNA linker that can be cleaved upon DNase addition. Due to the size, cost, convenience and ability to target and separate distinct cell populations, the microbeads were selected for cell-bead signaling studies.

The Notch functionalized microbead system exploits the biotin-streptavidin and antigen-antibody binding to present the Notch ligand in a highly directional fashion.

Streptavidin on the surface of the microbeads binds to a biotinylated mouse antibody raised against a histidine tag. Next, recombinant DLL4 that is histidine tagged binds to the antibody such that the activity of the DLL4 is not affected. To ensure consistent and effective DLL4 signaling, it is important to saturate the surface of the beads with the biotinylated antibody such that the concentration of DLL4 can be titrated to achieve the desired effect and study the effect of concentration. Although the beads were purchased from a commercial vendor with recommended bead-protein binding conditions, optimization was necessary due to the unique application and complex protein binding design (consisting of antibody and protein binding onto bead) entailed.

First, increasing amounts of biotinylated anti-6x HIS antibody including 10, 20, 50, 100, 200, 400, 600, 800, 1000, 1500, 2000, and 2100 ng per 10^7 beads at a concentration of 80000000 beads/ml were incubated with microbeads, stained with streptavidin-PE and analyzed for mean fluorescence intensities (MFI) using flow cytometry analysis. In preliminary experiments, concentrations ranging from 100 to 2100 ng per 10^7 beads were being used due to company recommended concentrations of 0.2 – 2 μg per 10^7 beads. When very little differences in fluorescence and lack of saturation in MFI were observed repeatedly, as shown in **Figure 3.2**, lower concentrations of protein per beads were then utilized. An optimum MFI was consistently found at a concentration of 100 ng per 10^7 beads with a homogeneous distribution of antibodies on beads (as indicated by the narrow peak), as shown in **Figure 3.3**, and was used for all subsequent bead studies. Statistical significance was found for 100 ng per 10^7 beads concentration after performing the Student's *t* test for all concentrations ($p<0.05$).

Bead concentration and incubation time were then varied and examined for optimal antibody immobilization onto bead. Bead concentrations of 80000000 beads/ml,

800000 beads/ml, and 8000 beads/ml were incubated with 100 ng of antibody per 10^7 beads for 30 min, 60 min, and 90 min, stained and analyzed for flow cytometry analysis. **Figure 3.4** indicates optimum MFI for bead concentration of 80000000 beads/ml with no statistical significance observed when comparing increased incubation times. However, increases in MFI were apparent for lower concentrations with time although statistically significant differences were only observed for 800000 beads/ml concentration between 30 min and 60 min ($p<0.05$) and 8000 beads/ml concentration between 30 min and 90 min ($p<0.05$) conditions using a Student's *t* test. As expected, more dilute bead concentrations resulted in lower MFIs for equivalent incubation times with 80000000 beads/ml found to be statistically significant for all three incubation times ($p<0.05$). Unless otherwise noted, all subsequent studies used an anti-6x HIS antibody coating of 100 ng per 10^7 beads for 30 min at 80000000 beads/ml.

3.3.2 Optimal conditions for DLL4 functionalization to microbeads

To assess the efficiency of the ligand functionalization process, we performed preliminary studies using immunofluorescent staining and flow cytometry analysis. DLL4 functionalized beads were stained with anti-DLL4 antibody and a FITC anti-rat IgG for ligand detection. Streptavidin coated beads were used as controls. As shown in **Figure 3.5**, 65% ± 6.53% of the beads were functionalized with DLL4 while uncoated control and biotinylated anti-6X HIS antibody coated beads showed 11.5% ± 0.57% and 8.67% ± 1.43% staining most likely due to nonspecific binding.

Functionalized beads were also stained and imaged through confocal microscopy to ensure the DLL4 ligand was immobilized around the entire surface of the bead. Functionalized beads were stained with rat anti-DLL4 antibody and an APC conjugated goat anti-rat antibody, air-dried and mounted for confocal microscopy. Beads were

imaged using transmission, reflection and fluorescent light at an excitation wavelength of 633 nm. As shown in **Figure 3.6F and 3.6H**, there seems to be a clear DLL4 coating (indicated by the yellow arrows) on the surface of the functionalized beads and not the anti-6x HIS antibody coated beads (shown in **Figure 3.6B and 3.6D**), despite the high autofluorescence. Photobleaching effects were most likely responsible for the inability to visualize DLL4 around the entire circumference. Confocal reflection microscopy also revealed the dispersed nature of the magnetic particles throughout the beads as seen in **Figure 3.6A and 3.6E**. Functionalized beads without the DNA linker (Bangs Labs) were also assessed for the integrity of DLL4 surface coating. These beads were stained with rat anti-DLL4 antibody and a FITC conjugated anti-rat antibody, air-dried and mounted for confocal microscopy, similar to the Biotin Binder Kit beads. Beads were imaged using transmission, reflection and fluorescent light at an excitation wavelength of 633 nm. **Figure 3.7F and 3.7H** show an intact coating of the complete surface on functionalized beads and the absence of one on anti-6x HIS antibody coated beads (shown in **Figure 3.7B and 3.7D**). Reflection microscopy shows a similar distribution of magnetic particles throughout these beads as well (**Figure 3.7A and 3.7E).**

Next, DLL4 was optimized similarly to anti-6x HIS antibody coating with the additional quantification of immobilized DLL4 molecules on Biotin Kinder Kit bead surfaces. Here, increasing amounts of DLL4 were coated onto previously optimized anti-6x HIS antibody beads and stained for flow cytometry analysis. Amount of DLL4 included 10, 20, 50, 100, 200, 500, 1000, and 2000 ng of DLL4 per 10^7 beads at a concentration of 80000000 beads/ml. As shown in **Figure 3.8A**, at the 200 ng concentration, the bead surface began to saturate. The drop in fluorescence intensity at 500 ng concentration cannot be explained. To ensure maximum saturation, 1000 ng of DLL4 per 10^7 beads concentration was used for all future studies. Concentrations of 200

– 2000 ng of DLL4 per 10^7 beads were shown to be significantly significant when compared to 10-100 ng of DLL4 per 10^7 beads concentrations ($p<0.05$). **Figure 3.8B** is a representative histogram for 1000 ng of DLL4 per 10^7 beads concentration. When quantified using the Quantum Simply Cellular (QSC) Kit, the average mean soluble equivalent fluorophores (MSEF) values ranged from 5000 – 8500. The average number of MSEF at saturation was 6830.665 DLL4 molecules / bead ± 711.2455 (mean ± SEM) from two independent experiments. Proactive® streptavidin coated beads were also evaluated in terms of DLL4 titration levels. These beads come with a company specific saturation capacity given for biotin-FITC binding. This value was converted for our system using the molecular weights of biotin-FITC and biotinylated anti-6x HIS antibody. Next, the Proactive® beads were coated with the calculated amount of anti-6x HIS antibody using the manufacturer's recommendation. For the DLL4 titration studies, concentrations of DLL4 including 20, 40, 100, 200, 400, 600, 1000, 2000, and 4000 ng of DLL4 per million beads were all incubated with beads. Coated beads were then blocked and stained for surface functionalized DLL4. The QSC Kit also enabled the quantification of the number of DLL4 molecules on the surface of beads. **Figure 3.9A** indicates the increase in MSEF with increasing DLL4 concentrations while **Figure 3.9B** shows a representative histogram for the 2000 ng of DLL4 per million beads concentration. Although the surface of the beads does not seem to be saturated, in the interest of cost, 2000 ng of DLL4 per million beads was used for all subsequent studies unless otherwise noted. The average number of MSEF for 2000 ng of DLL4 per million beads was 90058.38 DLL4 molecules / bead ± 3408.598 (mean ± SEM) from two independent experiments. This value is significantly larger than the 6830.665 DLL4 molecules found for the Biotin Binder Kit but seems reasonable due to the difference in

diameter (5.91 μm for Proactive® and 4.5 μm for Biotin Binder Kit) and the additional steric hindrance that in the Biotin Binder Kit due to the presence of a DNA linker.

An indirect DLL4 quantification method was also performed using an ELISA-based assay to quantify the amount of DLL4 on the Biotin Binder Kit beads by subtracting the measured amount of DLL4 on the wash buffers from the original amount of DLL4 added to the beads. The results indicated that approximately 130 ng of DLL4 was conjugated on 10^6 microbeads. Converted to molecules, this gives an estimate of ~1400 molecules/bead. This estimate is on the same scale as the direct estimate given above but is most likely underestimated due to the indirect nature of the assay and loss of protein that may have occurred due to inadequate washing.

Bead concentration and incubation time optimization studies resulted in similar optimized conditions found for the anti-6x HIS antibody coating. Bead concentrations of 80000000 beads/ml, 800000 beads/ml, and 8000 beads/ml were incubated with 1000 ng of DLL4 per 10^7 beads for 30 min, 60 min, and 90 min, stained and analyzed for flow cytometry analysis. **Figure 3.10** indicates a clear reduction in functionalization with decreased concentration and increased incubation time. A statistically significant difference was found when comparing lower concentrations for each incubation time for all concentrations except for the 80000000 beads/ml compared to 800000 beads/ml at 90 min. Although a statistically significant difference was only found for 800000 beads/ml concentration between 30 min and 60 min conditions ($p<0.05$) and 8000 beads/ml concentration between both 30 min and 60 min and 30 min and 90 min ($p<0.05$) using a Student's *t* test, the obvious trend appeared to be increased incubation time resulting in decreased coating efficiency. In the interest of cost and to make sure all sites were in fact saturated, beads were coated with 1000 ng of DLL4 per 10^7 beads for 30 min at concentrations of 80000000 beads/ml subsequently, unless otherwise noted.

3.3.3 Bead stability is compromised with the presence of DNA linker

After bead characterization and optimization, we wished to characterize the stability of the DLL4 ligand on the surface of the bead in its native storage buffer (0.1% BSA in PBS for Biotin Binder Kit and PBS for Proactive® beads) and medium. Initially, we hypothesized that serum may advantageously affect the bead stability. As a result, we tested serum-supplemented and serum-free media. First, immunofluorescence and flow cytometry analysis was used to assess the stability of the Biotin Binder Kit beads in their native storage conditions, 0.1% BSA in PBS at 4°C after 1 day and 6 days of storage (beads were coated with 100 ng of anti-6x HIS antibody and 1000 ng of DLL4 per 10 million beads). For the simplicity's sake, these experiments did not utilize the QSC kit, only differences in mean fluorescence intensity. Preliminary studies indicated a 70% reduction in amount of immobilized DLL4 on surface of beads, which implied an unstable bond, as shown in **Figure 3.11**. Further studies, albeit preliminary in nature, were then carried out where the effect of serum, buffer and medium were evaluated both at 4°C and 37°C after one hour of incubation. These studies revealed little DLL4 functionalized on surface of beads even at native conditions (0.1% BSA in PBS, 4°C), as shown in **Figure 3.12**. Nevertheless, except for the 0.1% BSA in PBS at 37°C and serum-supplemented medium at 4°C, no DLL4 can be detected on the surface of the beads after the incubation. Although no conclusive statement can be made due to the preliminary nature of the study, it appears that the DLL4 ligand is not effectively bound and that the PBS based buffer may be the most ideal for the preservation of the ligand on the bead. Also, it appears that the increased temperature interferes with the stability of the ligand, namely one of the bonds that is involved in the ligand functionalization.

Ligand stability was far superior for Proactive® beads. Immunofluorescence and flow cytometry analysis were again used to assess the stability of the beads, this time after 1 hr, 1 day and 1 week of incubation in PBS (both at 4°C and 37°C, serum-free medium and serum-supplemented medium (at 37°C only). Beads were coated with a predefined amount of anti-6x HIS antibody and 700 ng of DLL4 per million beads prior to incubation. Anti-6x HIS antibody beads were used as negative controls. As shown in **Figure 3.13**, even after 1 week of incubation in PBS, serum-free medium or serum-supplemented medium, functionalized beads retained a sizable percentage of DLL4 on the surface. The decreases in DLL4 amounts compared to the suggested storage conditions (4°C PBS) and as a function of time are summarized in **Tables 3.1 and 3.2**, respectively with values representing amount left on surface. In terms of best buffers for storage, 4°C PBS appears to be the best, although after 1 week of incubation there seems to be little difference between temperatures for the PBS buffer. Serum-free medium appeared to least preserve the original surface density of DLL4 after one week of culture. Thus, the presence of serum did not appear to be a factor in preserving the surface integrity. Interestingly, there seems to be a 40-70% decrease in surface density of DLL4 after 23 hours of incubation.

3.3.4 Viability of C2C12 myoblasts and embryonic stem cells is not significantly affected by the presence of beads

Polystyrene particles have been shown to display cytotoxic effects on white blood cells at high concentrations (Olivier et al., 2003). Thus, a cytotoxicity study of beads at varying concentrations and multiple timepoints with relevant cells was performed. The MTT assay is a common *in vitro* cytotoxicity assay that utilizes mitochondrial dehydrogenases to cleave the tetrazolium ring and produce visible crystals that can be

dissolved and spectrophotometrically measured. Due to its ease and accuracy, this assay was selected for our purposes. Since polystyrene beads were functionalized with DLL4, it was important to show that DLL4, itself, was not toxic at high concentrations (as compared to polystyrene beads). In the primary experiment, C2C12 myoblasts were incubated at bead to cell ratios of 0.1:1, 1:1 and 5:1 for 3 days, 7 days and 9 days. As shown in **Figure 3.14 and Table 3.3**, DLL4 functionalization of beads did not affect cell viability, although a statistically significant difference was seen between the 0.1:1 bead to cell ratio between both type of beads the 3 day timepoint. A similar study was carried out for R1 embryonic stem cells, where cells were incubated at bead to cell ratios of 0.1:1, 1:1 and 10:1 for 3 days, 5 days and 7 days. A similar trend was observed, as shown in **Figure 3.15**. A statistically significant difference was again seen for the 0.1:1 bead to cell ratio at the 3 day timepoint, as shown in **Table 3.3**. Viability does not appear to be affected in both cell types with the addition of beads, although a decrease in viability is apparent in the C2C12 with increased bead to cell ratios for the 7 day and 9 day timepoints. Interestingly, the beads appear to have a proliferative effect on the cells as evident by the >100% viability values in some conditions.

Once we established that DLL4 functionalization did not affect cell viability, we performed additional studies with uncoated beads only (in the interest of cost) at higher bead to cell ratios. **Figure 3.16** shows the reduced viability with increasing bead to cell concentration for all three timepoints. Although statistically significant differences were observed when comparing all bead to cell ratios for the 3 day timepoint (except when comparing 1:1 and 10:1), the maximum reduction in viability was a mere 10%, as shown in **Figure 3.16 and Table 3.4**. The later timepoints resulted in closer viability percentages with statistically significant differences when comparing both 1:1 and 10:1 to 40:1 bead to cell ratio, as observed in **Figure 3.16 and Table 3.4**. Even at a bead to cell

ratio of 40:1, the cell viability is nearly 100% at the week timepoint. For R1 cells, however, the results differed. An increased cytotoxicity for higher concentrations was observed for the 3 day timepoint only, as shown in **Figure 3.17**. Both 5 day and 7 day timepoints did not display such a trend for increasing bead to cell ratios (**Figure 3.17**). Statistical analysis confirmed a difference when comparing 0.1 and 1:1 to 10:1 and 40:1 for both 3 days and 5 days (**Table 3.5**). These results most likely reflect the high proliferation rate of embryonic stem cells that may mask any bead toxicity effects and would require repeat bead additions to truly assess the effect of high bead concentrations. Nevertheless, the lowest viability observed (7 day, 10:1 bead to cell ratio) was still 80% (**Figure 3.17**). Although some reduction in cell viability was observed, these results illustrate that the functionalized beads do not exhibit any serious cytotoxic effects for short-term cell bead studies in myoblasts and embryonic stem cells.

3.4 DISCUSSION

Magnetic beads have been widely used for cell separation and flow cytometry applications for many years (Egeland et al., 1991; Patel et al., 1995; Swann et al., 1992). From the cytocompatibility and manufacturing standpoints, beads such as these are especially attractive. More recently, however, beads in general have been used for a variety of applications including force measurement and application, ligand-receptor binding studies, signal localization and vesicle trafficking and T cell expansion and activation (Curtsinger et al., 1997; Fass et al., 2003; Friedlander et al., 2005; Ito et al., 2003; Kim et al., 2004; Li et al., 2005; Maus et al., 2002; Patel et al., 1995). In the area of T cell activation and expansion, beads have been particularly successful. For example, Levine and colleagues demonstrated up to 10^{11}-fold expansion in a 60-day culture of CD4+ T cells with anti-CD3 antibody and anti-CD28 antibody coated beads (Levine et

al., 1997). In another study, Ito et al found anti-CD3 and anti-CD28 antibody bound beads to be more effective than plate immobilized antibodies in promoting T cell activation and IL-2 secretion (Ito et al., 2003). These findings demonstrate the promise and potential in such a bead platform for providing antibody or protein based signals to cells.

Significant strides have been made in the field of T cell development due to the generation of a Notch ligand DLL1 expressing stromal cell line, OP9-DL1, by Zuniga-Pflucker and colleagues (Zuniga-Pflucker 2004). Several studies have successfully demonstrated T cell development to the DP stage from both murine and human cord blood and bone marrow-derived hematopoietic progenitors using the stroma derived and Notch signals from the OP9-DL1 cell line (Murine embryonic stem cells have also been shown to differentiate to the T cell lineage) (De Smedt et al., 2004; La Motte-Mohs et al., 2005; Schmitt et al., 2002, Schmitt et al., 2004). Other groups have relied on plate immobilized Notch ligands to provide the necessary Notch signaling for T cell development (Dallas et al., 2005; Delaney et al., 2005). (Notch ligands have been shown to require immobilization for effective signaling (Varnum-Finney et al., 2000).) Immobilized ligands offer the ability to quantitatively control the amount of Notch signaling the seeded population receives, an advantage the OP9-DL1 system does not provide. Although this system has demonstrated promise in T cell commitment ex vivo in a cell-free manner, the expense and stability of the plate-adsorbed ligand are legitimate concerns for long-term culture.

Originally, we envisioned a microbead with a Notch ligand directionally functionalized on its surface; in this way, the bead had the potential to be separated from its target cell and provide the necessary signaling in a dose-responsive manner. For this reason, we first used streptavidin-coated microbeads that included a DNA linker for

bead-cell separation (with the addition of DNase). The magnetic quality of the beads was an added feature that could aid in specific cell populations. Such a large diameter of the beads ensured prevention of endocytosis.

First, Biotin Binder Kit streptavidin coated microbeads were functionalized with a Notch ligand using a biotinylated anti-HIS antibody and a HIS tagged recombinant Delta like ligand 4. Such microbeads have been used in T cell culture studies, but only for activation applications (Maus et al., 2002; Trickett et al., 2002). Our approach utilized a highly directional binding scheme through streptavidin-biotin binding and antigen-antibody interactions that is performed under mild conditions which does not affect the binding site of the ligand and should ensure appropriate confirmation for efficient Notch signaling. Preliminary physical characterization of these beads using flow cytometry and confocal microscopy indicated ~65% functionalization efficiency and surface immobilized DLL4.

Next we performed in depth optimization studies of protein functionalization to the surface of beads, examining parameters such as protein concentration (for both anti-6x HIS antibody and DLL4), incubation time and bead concentration. These studies revealed similar conditions for optimum antibody/ligand functionalization. The extent of functionalization reached its optimum with the highest bead concentration test, 80000000 beads/ml, and the shortest incubation time, 30 min. This seems somewhat intuitive in that dilute bead concentrations would hinder adequate interaction between the protein and microbead. For the more dilute bead concentrations, longer incubation times did result in increased protein binding, albeit less than the optimum. It is perhaps likely that longer incubation periods may result in increased levels of bound protein. Curtsinger and colleagues demonstrated a rapid immobilization time of 20-30 min for MHC1 protein functionalized sulfate polystyrene latex microspheres, a time period that is similar to

ours. Additionally, the group reported a loss in surface density for extended incubation times, perhaps due to loss or protein from surface (Curtsinger et al., 1997). For protein titration studies, biotinylated anti-6x HIS antibody appeared to saturate the surface at only 100 ng/10^7 beads while the DLL4 ligand saturated at a 10-fold higher concentration, 1000 ng/10^7 beads. Steric hindrance and sizes of the protein (where anti-6x HIS antibody has a molecular weight of near 150 kDa and DLL4 has a molecular weight of 55 kDa) are speculated to play a role in these observed results.

Quantification of bound DLL4 molecules was performed using two methods, an indirect ELISA on the washes performed during DLL4 bead fabrication and a direct fluorescent based measurement that translated to specific molecules due to a calibration curve from commercially available calibrated standard beads. An estimated amount of surface saturation can be assessed from the following equation (Bangs Labs, 1999):

$$\mathbf{S = 6/(\rho_s * d) * C}$$

where:

S = amount of representative protein required to achieve surface saturation (mg protein/g microspheres)

$\mathbf{\rho_s}$ = density of solid sphere (g/cm^3)

d = mean diameter (μm)

C = capacity of microsphere surface for a given protein (mg protein/m^2 of sphere surface)

Using a diameter of 4.5 μm, density of 1.05 g/cm^3 for polystyrene beads, capacity of 3 mg/m^2 for DLL4 and a capacity of 2.5 mg/m^2 for biotinylated anti-6x HIS antibody and bead concentration of 4x10^8 beads/1.6g (company communication), we found ~50000

antibody molecules/bead and 166000 DLL4 molecules/bead. ELISA analysis indicated 130 ng of DLL4 per one million beads which translates to ~1400 molecules per bead. The indirect nature of assay and the high possibility of losing protein during the process prompted us to try a more direct method of quantification namely the calibration beads. Using this method, we observed nearly 6800 molecules per bead, an estimate that is nearly 5 times greater than the ELISA method but on the same scale. Both values fall well below the calculated saturation limit.

Next, we wished to qualitatively assess the degree of functionalization on the entire bead surface using confocal microscopy. Due to the polystyrene nature of the beads, autofluorescence was evident at lower excitation wavelengths and was reduced at an excitation wavelength of 633 nm (Gorostiza et al., 2005). Regardless, a circumferential surface coating seemed apparent from fluorescent images on functionalized microbeads in comparison to the uncoated microbeads, indicating successful DLL4 functionalization. Photobleaching most likely reduced the fluorescence around the entire bead. Images acquired using the reflectance mode of the confocal microscope illustrated the dispersed placement of the magnetite particles in the beads, which are known to be reflective (Green et al., 2001).

Finally, ligand stability on bead surface in 0.1% BSA in PBS, serum-free medium and serum-supplemented medium at 4°C and 37°C was characterized using flow cytometry studies. Preliminary studies were conducted assessing the ligand density after 6 days of incubation in native storage conditions (0.1% BSA in PBS 4°C). The 70% reduction in surface ligand density suggested a weak bond and prompted us to conduct further studies. We hypothesized that the presence of serum interfered with one of the bonds in the bead and thus performed a study where beads were incubated in PBS, serum-free medium and serum-supplemented medium at 4°C and 37°C. These studies,

were non ideal in nature due to low native ligand density. Nevertheless, studies revealed a substantial reduction in surface density for the PBS buffer when subjected to 37°C and the absence of surface ligand on all other conditions, with the presence of some ligand on serum-supplemented medium at 4°C, only. Regardless, these results disproved our serum hypothesis.

The Biotin Binder Kit beads we used depend on three bonds for effective DLL4 functionalization. First, the streptavidin is bound to the surface of the polystyrene beads through a DNA linker. The second bond consists of streptavidin-biotin binding which is known to have a bond strength of $K_a = 10^{15}$/M (Bangs Labs 1999). The final bond is comprised of antigen-antibody interactions $K_a = 10^{7}$-10^{11}/M (Bangs Labs 1999). Considering this, the DNA linker appeared to be the "weakest link." DNase, an enzyme that cleaves DNA, is known to be omnipresent and have a higher biological activity at 37°C. This led us to speculate that the DNA linker was in fact being cleaved and releasing the ligand.

To test this hypothesis, we performed similar characterization studies on similar magnetic streptavidin coated beads that lacked the DNA linker. These beads were 5.91 μm in diameter with a saturation capacity for biotin-FITC given for each lot. We converted this saturation capacity to our biotinylated anti-6x HIS antibody based on molecular weight differences and used this amount to coat the beads. For the DLL4 functionalization, we tested a range of values from 20, 40, 100, 200, 400, 600, 1000, 2000, and 4000 ng of DLL4 per million beads. Our results indicated a linear relation without saturation for the concentrations tested. For cost-related issues, we used 2000 ng of DLL4 per million for subsequent studies unless otherwise noted. This concentration corresponded to an average 90058.38 DLL4 molecules / bead ± 3408.598 (mean ± SEM), deduced from QSC kit calibration beads. Using the equation given above, the saturation

capacity for the 5.91 μm beads is 2.9 mg per gram of beads which corresponds to virtually 3700000 DLL4 molecules per bead. Our empirically derived number of DLL4 molecules is well below this value. Our titration results suggest an increased capacity for DLL4 functionalization, as well. Confocal fluorescence and reflection microscopy confirmed successful DLL4 functionalization on the bead surface with a dispersion of magnetic particles throughout the inside of the bead, similar to the Biotin Binder Kit beads. Finally, we conducted stability studies on beads, similarly to the described methods above with additional 1 day and 1 week timepoints and PBS usage (instead of 0.1% BSA in PBS). These results were far more encouraging with some DLL4 present in all conditions even after 1 week of incubation. The nearly 50% reduction in surface density of DLL4 under its native storage conditions (PBS 4°C) after one day of incubation is troublesome and requires further study. Regardless, the absence of the DNA linker resulted in more stable DLL4 functionalization for an extended period of time in serum supplemented and serum-free medium.

Cytotoxicity of beads was also examined in this study. Beads have been shown to be cytotoxic to cells at various concentrations. For example, Olivier and colleagues performed a study where they assessed the cytotoxic effect of polystyrene and alumina beads with different sizes and concentrations on macrophages and fibroblasts. Increased size and concentrations were found to be the most detrimental to cell viability for both types of beads and cells (Olivier et al., 2003). To assess the cytotoxicity of our beads, we first compared DLL4 functionalized and uncoated beads at various bead to cell ratios for C2C12 myoblasts and R1 embryonic stem cells. We found no statistical difference between bead to cell ratios for multiple timepoints for both cell types (Day 3, 0.1:1 was the sole exception). This led us to further studies of high bead to cell concentrations with both cell types. Our results show a similar trend to Olivier and colleagues with reduced

viability in myoblasts and stem cells (for early time points only) at increasing bead to cell concentrations. No obvious trend was apparent in the stem cell viability results at later time points most likely due to high proliferation rate. The maximum reduction in viability for both cell types was a mere 10-20%, displaying minimal cytotoxic effects of beads. These results indicate that DLL4 functionalized beads and the magnetic beads in general do not pose any serious cytotoxic threats for stem cells and myoblasts.

In this study, we have successfully optimized the functionalization of DLL4 ligand onto magnetic streptavidin coated microbeads using antigen-antibody and biotin-streptavidin interactions. Furthermore, we have demonstrated qualitatively and quantitatively the presence of DLL4 on the beads for up to a week in serum free and serum supplemented medium under physiologic conditons. We have extensively characterized the non-toxic nature of the bead in both myoblasts and embryonic stem cells, as well. Our studies suggest the unstable nature of a DNA linker in such a functionalized bead and warrant further study.

Table 3.1 Reduction in surface functionalized DLL4 microbead as compared to original amount. (values represent percent)

	1 hr	**1 day**	**1 week**
Conditions			
PBS 4ºC	n/a	46.81	38.81
PBS 37ºC	66.25	43.76	22.13
Serum-free medium 37ºC	43.30	23.21	3.47
Serum-supplemented medium 37ºC	59.18	19.79	10.41

Table 3.2 Reduction in surface functionalized DLL4 microbead as a function of time. (values represent percent)

	1 hr	1 day	1 week
Conditions			
PBS 4ºC	n/a	46.81	38.81
PBS 37ºC	n/a	66.05	33.41
Serum-free medium 37ºC	n/a	53.59	8.02
Serum-supplemented medium 37ºC	n/a	33.44	17.60

Table 3.3 Student's *t* test analysis of statistical difference between uncoated and DLL4 functionalized beads cytotoxicity in C2C12 myoblasts and R1 embryonic stem cells: *p* values

	0.1:1	**1:1**	**5:1 (C2C12) or 10:1 (R1)**
C2C12 cells			
3 Day	***<0.05***	>0.05	>0.05
7 Day	>0.05	>0.05	>0.05
9 Day	>0.05	>0.05	>0.05
R1 cells			
3 Day	***<0.05***	>0.05	>0.05
5 Day	>0.05	>0.05	>0.05
7 Day	>0.05	>0.05	>0.05

Table 3.4 Student's *t* test analysis of cytotoxicity differences from differences in bead to cell ratios for uncoated beads with C2C12 myoblasts: *p* values

	1:1	**10:1**	**20:1**	**40:1**
3 Day				
0.1:1	***<0.05***	***0.055***	***<0.05***	***<0.05***
1:1	-----	>0.05	***<0.05***	***<0.05***
10:1	------	-----	***<0.05***	***<0.05***
20:1	-----	-----	-----	***0.055***
5 Day				
0.1:1	>0.05	>0.05	>0.05	***<0.05***
1:1	-----	>0.05	***<0.05***	***<0.05***
10:1	-----	-----	***<0.05***	***<0.05***
20:1	-----	-----	-----	>0.05
7 Day				
0.1:1	>0.05	>0.05	>0.05	>0.05
1:1	-----	>0.05	>0.05	***<0.05***
10:1	-----	-----	>0.05	***<0.05***
20:1	-----	-----	-----	>0.05

Table 3.5 Student's *t* test analysis of cytotoxicity differences from differences in bead to cell ratios for uncoated beads in R1 cells: *p* values

	1:1	**10:1**	**20:1**	**40:1**
3 Day				
0.1:1	>0.05	***0.054***	>0.05	***<0.05***
1:1	-----	***0.050***	>0.05	***<0.05***
10:1	-----	-----	>0.05	>0.05
20:1	-----	-----	-----	>0.05
5 Day				
0.1:1	>0.05	***<0.05***	>0.05	***<0.05***
1:1	-----	>0.05	>0.05	***<0.05***
10:1	-----	-----	>0.05	***<0.05***
20:1	-----	-----	-----	>0.05
7 Day				
0.1:1	***<0.05***	>0.05	>0.05	>0.05
1:1	-----	>0.05	>0.05	>0.05
10:1	-----	-----	>0.05	>0.05
20:1	-----	-----	-----	>0.05

Figure 3.1 Schematic of Notch functionalized microbead. (A) with and (B) without DNA linker. Figure is not drawn to scale and has been simplified to better illustrate the structure.

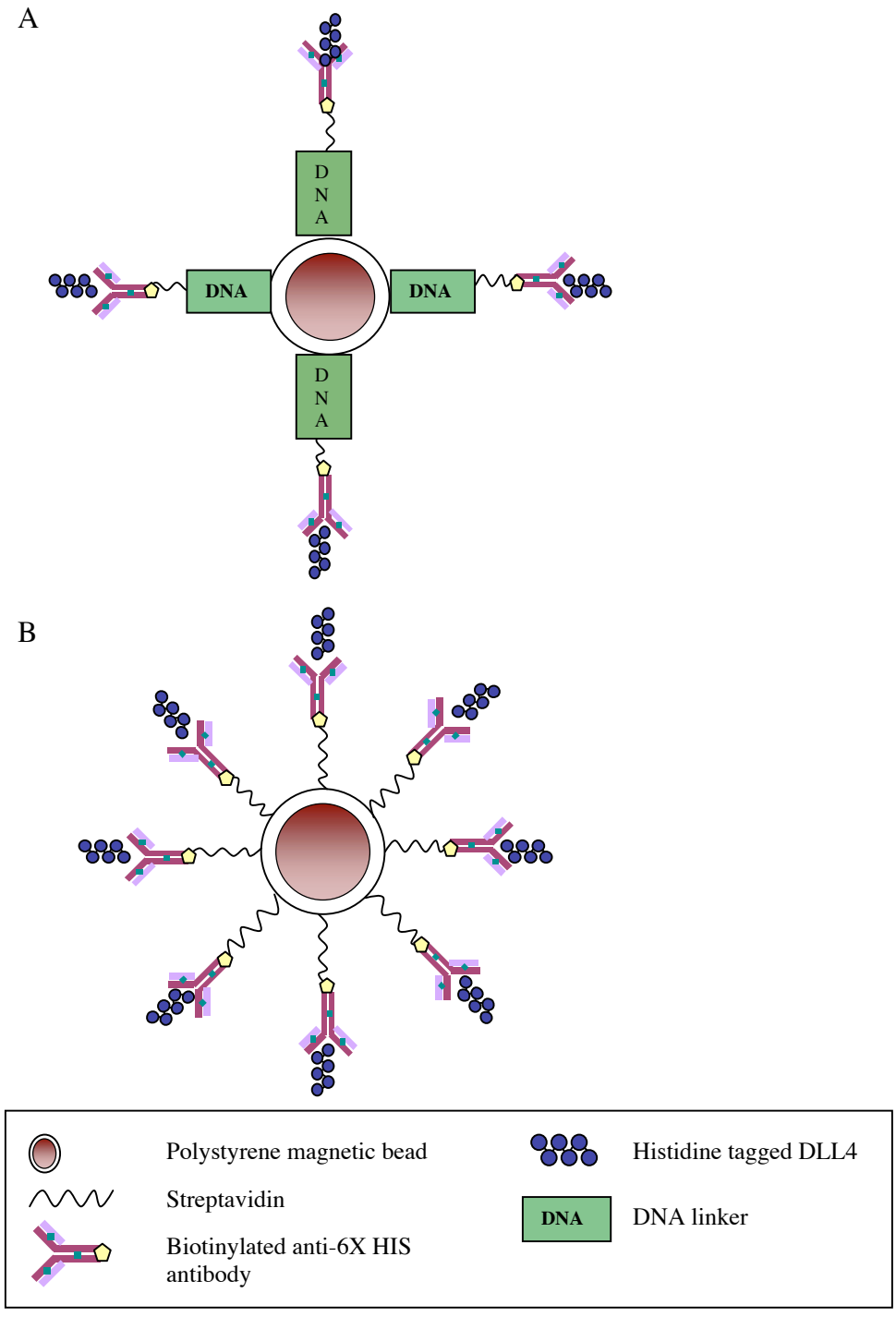


Figure 3.2 Anti-6x HIS antibody saturation of magnetic beads for high concentrations using flow cytometry analysis. Biotin binder kit streptavidin coated beads were coated with various concentrations of anti-6x HIS antibody and analyzed using flow cytometry analysis. Beads were blocked in 3% BSA in PBS and stained with 0.25 μg of streptavidin-PE /million beads. Average mean fluorescence intensity values are given below with bars indicating standard error. Uncoated beads served as negative controls. *denotes statistical significance when comparing 100 ng of anti-6x HIS antibody/10 million beads to all other concentrations using a one tailed Student's *t* test ($p<0.05$)

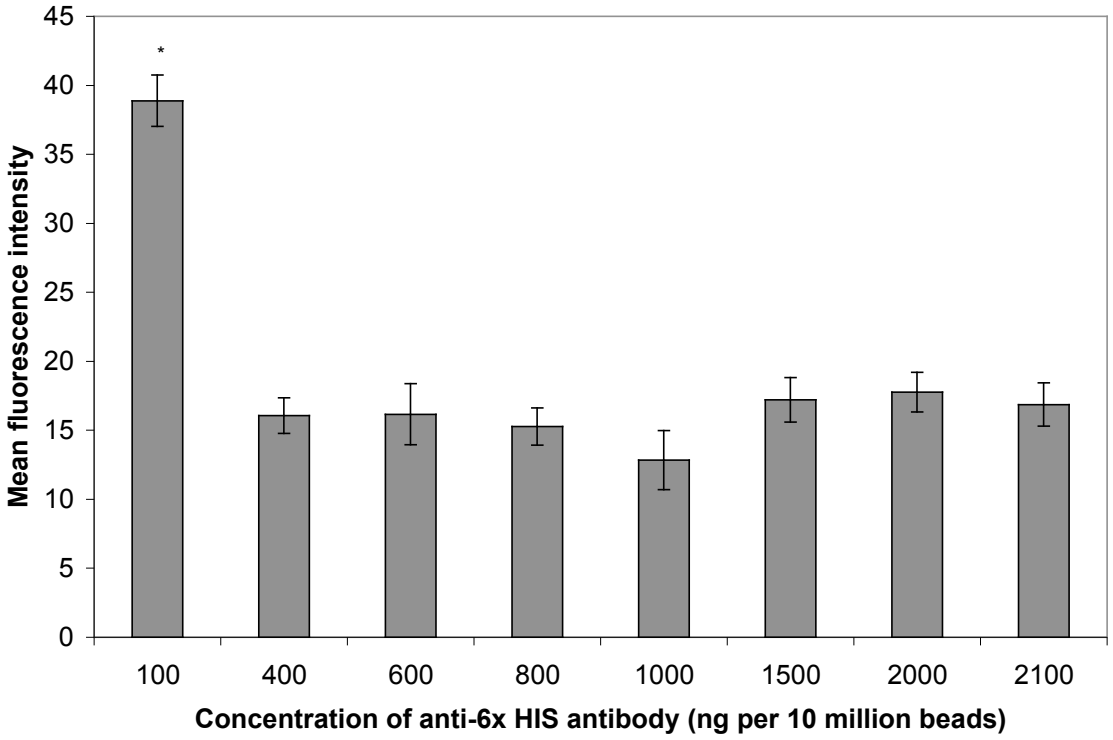
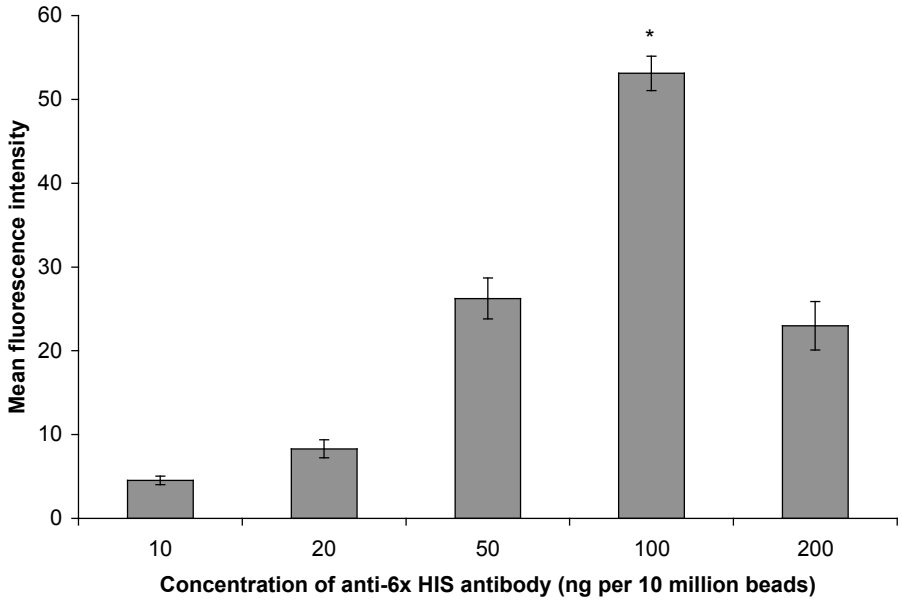


Figure 3.3 Anti-6x HIS antibody saturation of magnetic beads using flow cytometry analysis. (A) Titration of antibody concentrations represented as average mean fluorescence intensities. (B) Representative histogram of 100 ng/10 million beads concentration. Biotin binder kit streptavidin coated beads were coated with various concentrations of anti-6x HIS antibody and analyzed using flow cytometry analysis. Beads were blocked in 3% BSA in PBS and stained with 0.25 μg of streptavidin-PE /million beads. Average mean fluorescence intensity values are given below with bars indicating standard error. Uncoated beads served as negative controls. *denotes statistical significance when comparing 100 ng of anti-6x HIS antibody/10 million beads to all other concentrations using a one tailed Student's *t* test ($p<0.05$)

A



B

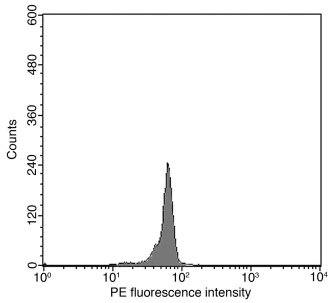


Figure 3.4 Bead concentration and incubation period optimization for anti-6x HIS antibody coating using flow cytometry analysis. Biotin binder kit streptavidin coated beads were incubated in 80000000, 800000, and 8000 beads/ml concentrations for 30, 60, and 90 min incubation periods at 100 ng of anti-6x HIS antibody/10 million beads. Beads were blocked in 3% BSA in PBS and stained with 0.25 μg of streptavidin-PE /million beads. Average mean fluorescence intensity values are given below with bars indicating standard error. Uncoated beads served as negative controls.

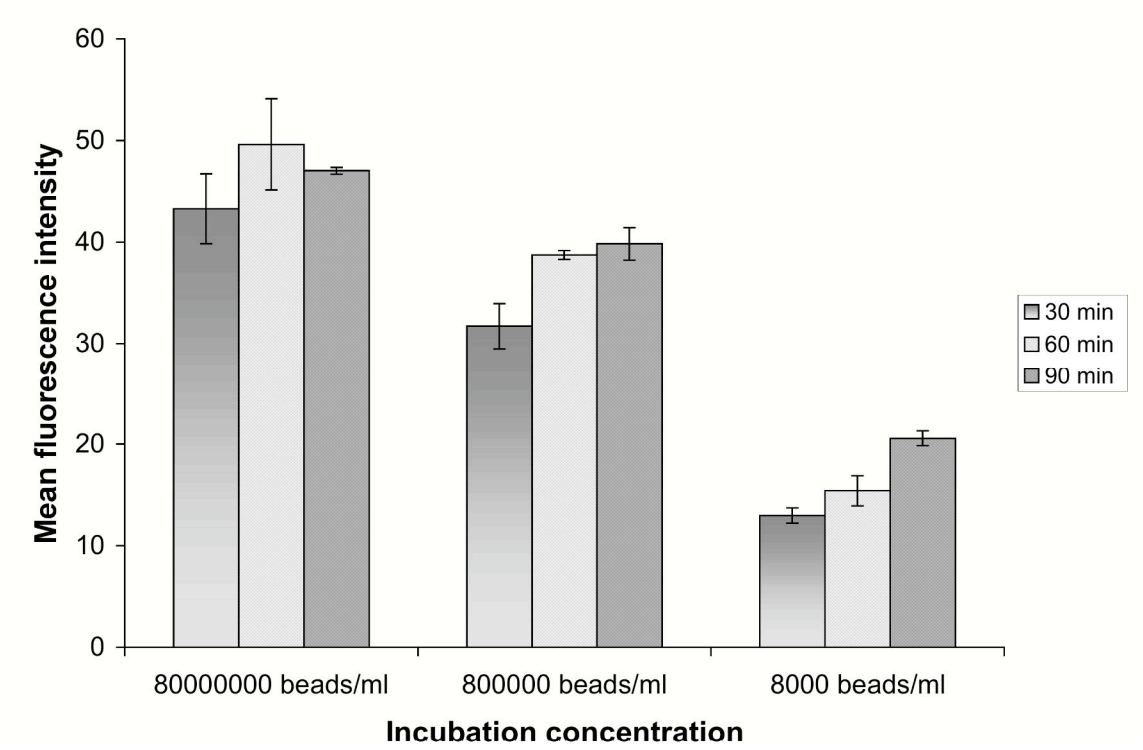


Figure 3.5 Microbeads can be efficiently functionalized with Notch ligand DLL4. To assess the efficiency of ligand binding, functionalized beads were stained with anti-DLL4 antibody and FITC anti-rat IgG antibody and analyzed using flow cytometry. Streptavidin coated beads and biotinylated anti-6x HIS tagged antibody coated beads were used as negative controls. A. Solid histogram represents uncoated beads while unfilled histogram represents DLL4 functionalized beads. B. Solid histogram represents biotinylated anti-6x HIS tagged coated antibody beads while unfilled histogram represents DLL4 functionalized beads. C. Comparison of coating efficiency for coated and uncoated beads. All experiments were performed in triplicate. * indicates $p < 0.05$ compared to both control bead populations using a Student's *t* test.

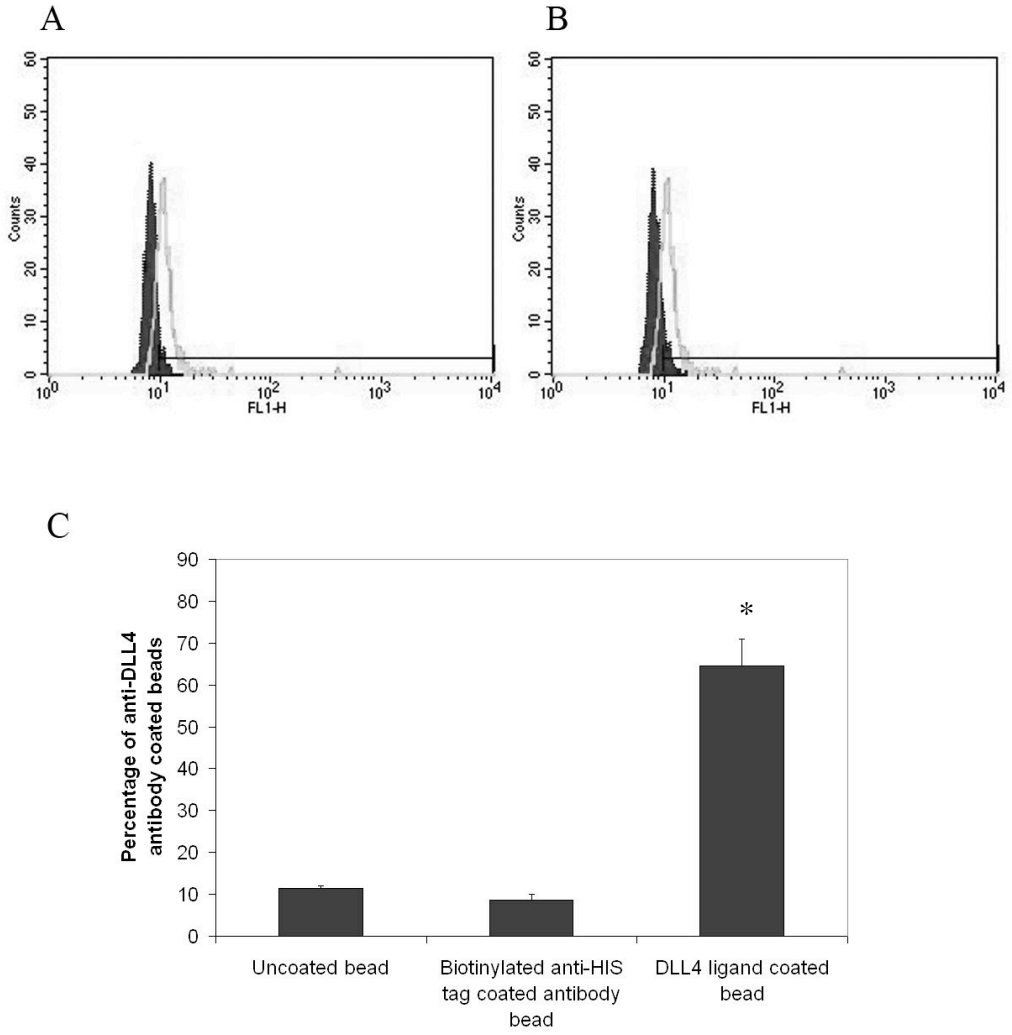


Figure 3.6 Fluorescence microscopy confirmation of functionalized DLL4 on the surface of Biotin Binder Kit beads. Anti-6x HIS beads and DLL4 functionalized beads were blocked and stained with anti-DLL4 antibody and APC anti-rat IgG antibody and visualized under reflection, confocal and transmission microscopy. Anti-6x HIS beads are shown below under reflection (A), confocal (B), and transmission microscopy (C). DLL4 functionalized beads are shown below under reflection (E), confocal (F), and transmission microscopy (G). (D) and (H) indicate overlays of reflection and fluorescence channels for anti-6x HIS beads and DLL4 functionalized beads, respectively. Yellow arrows indicate functionalized DLL4 on bead surface.

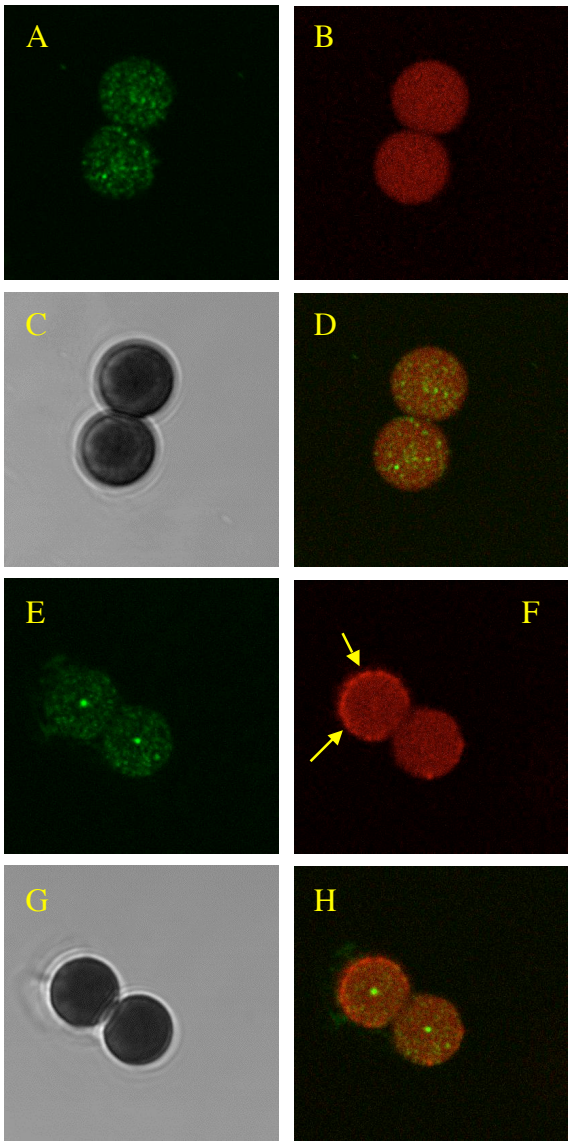


Figure 3.7 Fluorescence microscopy confirmation of functionalized DLL4 on the surface of Proactive® beads. Anti-6x HIS beads and DLL4 functionalized beads were blocked and stained with anti-DLL4 antibody and APC anti-rat IgG antibody and visualized under reflection, confocal and transmission microscopy. Anti-6x HIS beads are shown below under reflection (A), confocal (B), and transmission microscopy (C). DLL4 functionalized beads are shown below under reflection (E), confocal (F), and transmission microscopy (G). (D) and (H) indicate overlays of reflection and fluorescence channels for anti-6x HIS beads and DLL4 functionalized beads, respectively.

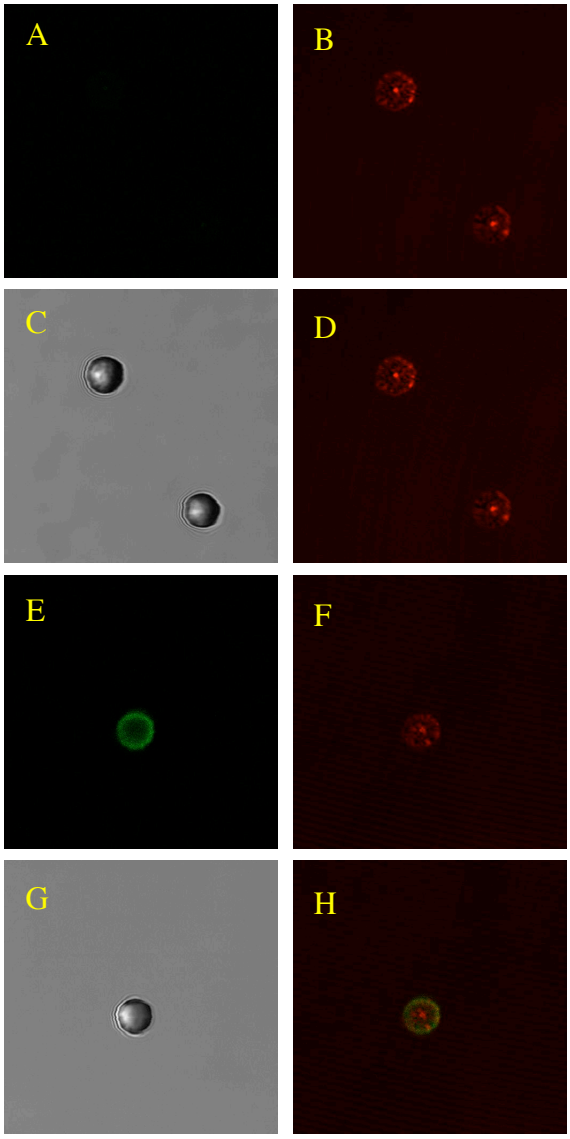
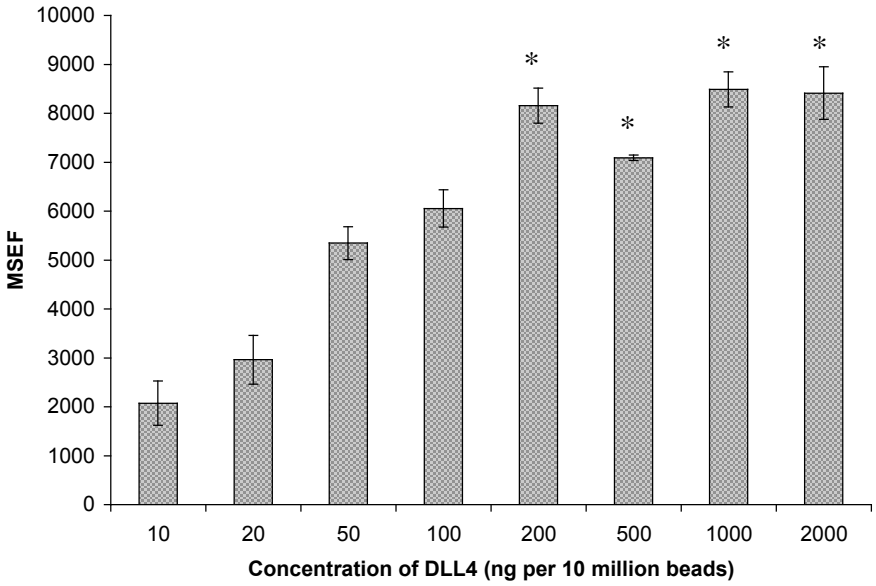


Figure 3.8 DLL4 saturation of magnetic beads using flow cytometry analysis. (A) Titration of DLL4 concentrations represented as average mean fluorescence intensities. (B) Representative histogram of 1000 ng/10 million beads concentration. Biotin binder kit DLL4 coated beads were coated with various concentrations of anti-6x HIS antibody and analyzed using flow cytometry analysis. Beads were blocked in 3% BSA in PBS and stained with anti-DLL4 antibody and FITC anti-rat IgG antibody. Average mean soluble equivalent fluorophore (MSEF) values are given below with bars indicating standard error. Anti-6x HIS antibody coated beads served as negative controls. *denotes statistical significance when comparing 200-2000 ng of DLL4/10 million beads to all other lower concentrations (10-100 ng of DLL4/10 million beads) using a one tailed Student's *t* test ($p<0.05$)

A



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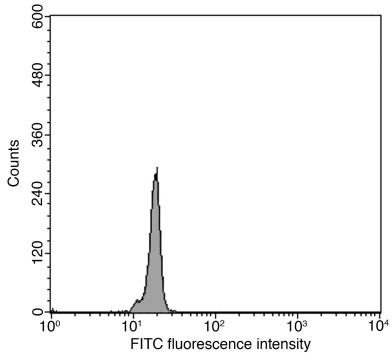
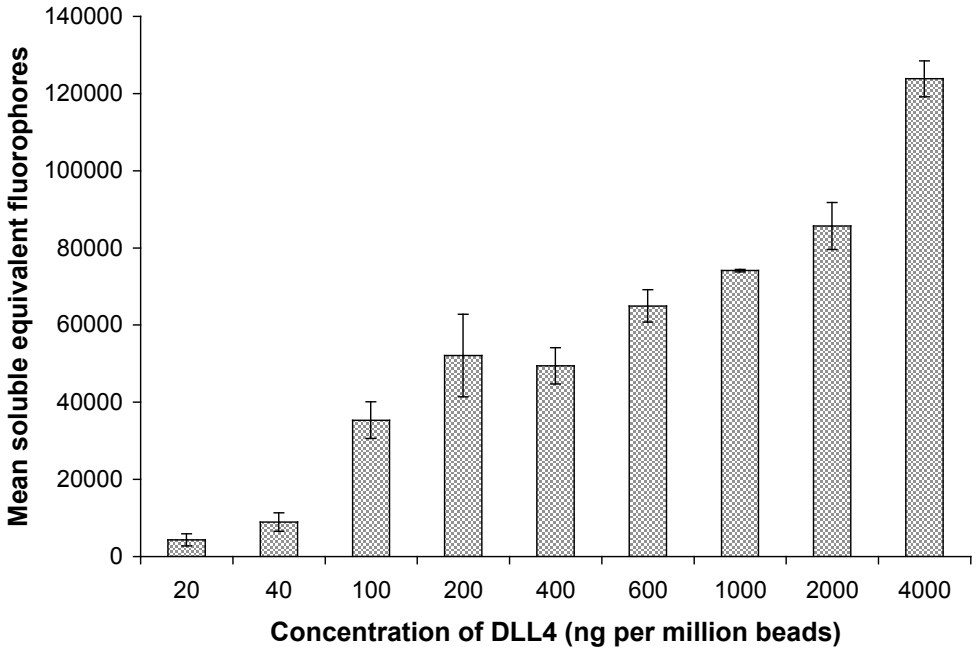


Figure 3.9 DLL4 saturation of Proactive ® magnetic beads using flow cytometry analysis. (A) Titration of DLL4 concentrations represented as average mean fluorescence intensities. (B) Representative histogram of 2000 ng/million beads concentration. Beads were coated with a predefined amount of anti-6x HIS antibody and varying concentrations of DLL4 and analyzed using flow cytometry analysis. Beads were blocked in 3% BSA in PBS and stained with anti-DLL4 antibody and FITC anti-rat IgG antibody. Average mean soluble equivalent fluorophore (MSEF) values are given below with bars indicating standard error. Anti-6x HIS antibody coated beads served as negative controls.

A



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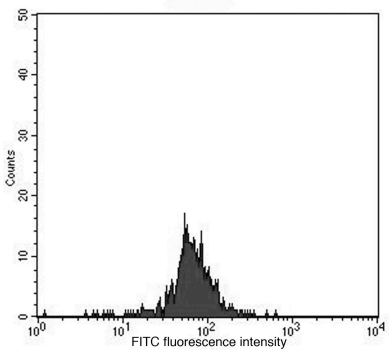


Figure 3.10 Bead concentration and incubation period optimization for DLL4 coating using flow cytometry analysis. Biotin binder kit streptavidin coated beads were incubated in 80000000, 800000, and 8000 beads/ml concentrations for 30, 60, and 90 min incubation periods at 100 ng of anti-6x HIS antibody/10 million beads and 1000 ng of DLL4/10 million beads. Beads were blocked in 3% BSA in PBS and stained with anti-DLL4 antibody and FITC anti-rat IgG antibody. Average mean fluorescence intensity values are given below with bars indicating standard error. Anti-6x HIS antibody coated beads served as negative controls.

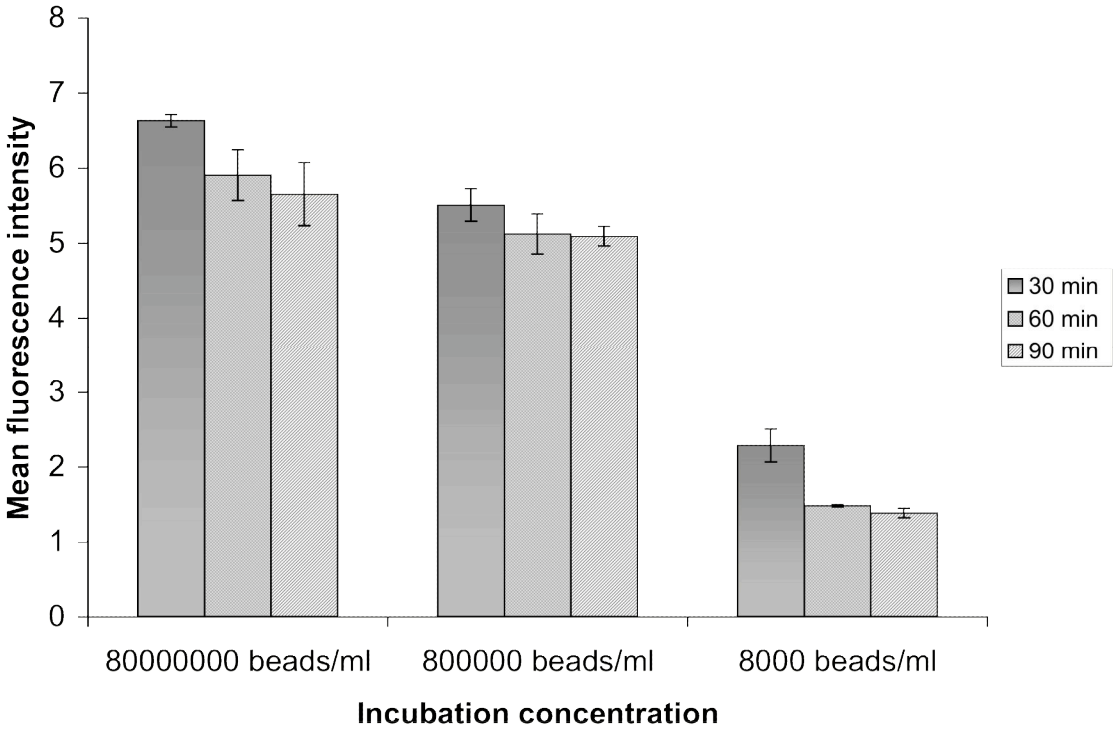


Figure 3.11 DLL4 functionalized Biotin Binder Kit bead stability after 6 days in native storage conditions. Beads were coated with 100 ng of anti-6x HIS antibody and 1000 ng of DLL4 per 10 million beads and incubated for one day and six days in 0.1% BSA in PBS at 4ºC. Following incubation, beads were stained and analyzed for surface DLL4 using flow cytometry analysis. Beads were blocked in 3% BSA in PBS and stained with anti-DLL4 antibody and FITC anti-rat IgG antibody. Average mean fluorescence intensity values are given below with bars indicating standard error. Anti-6x HIS antibody coated beads served as negative controls.

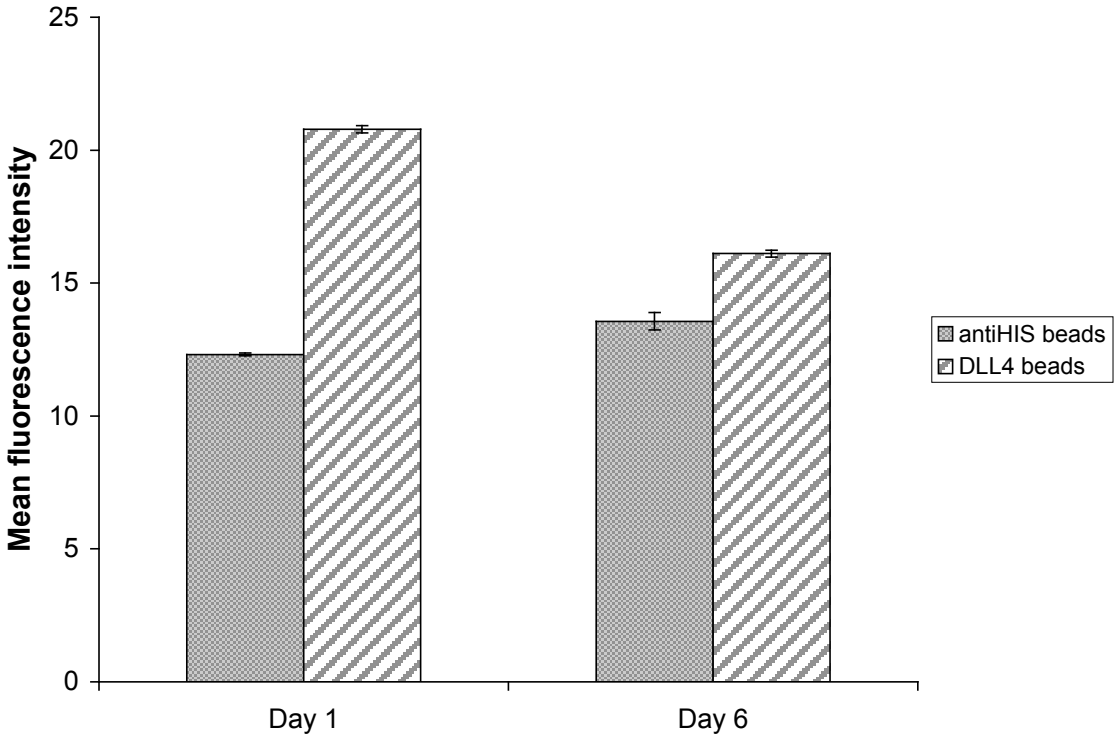


Figure 3.12 Effect of temperature, serum and medium on the stability of DLL4 functionalized Biotin Binder Kit bead after 1 hr incubation. Beads were coated with 100 ng of anti-6x HIS antibody and 1000 ng of DLL4 per 10 million beads and incubated for one hr in 0.1% BSA in PBS, serum-free medium and serum-supplemented medium at 4ºC and 37ºC. Following incubation, beads were stained and analyzed for surface DLL4 using flow cytometry analysis. Beads were blocked in 3% BSA in PBS and stained with anti-DLL4 antibody and FITC anti-rat IgG antibody. Average mean fluorescence intensity values are given below with bars indicating standard error. Anti-6x HIS antibody coated beads served as negative controls.

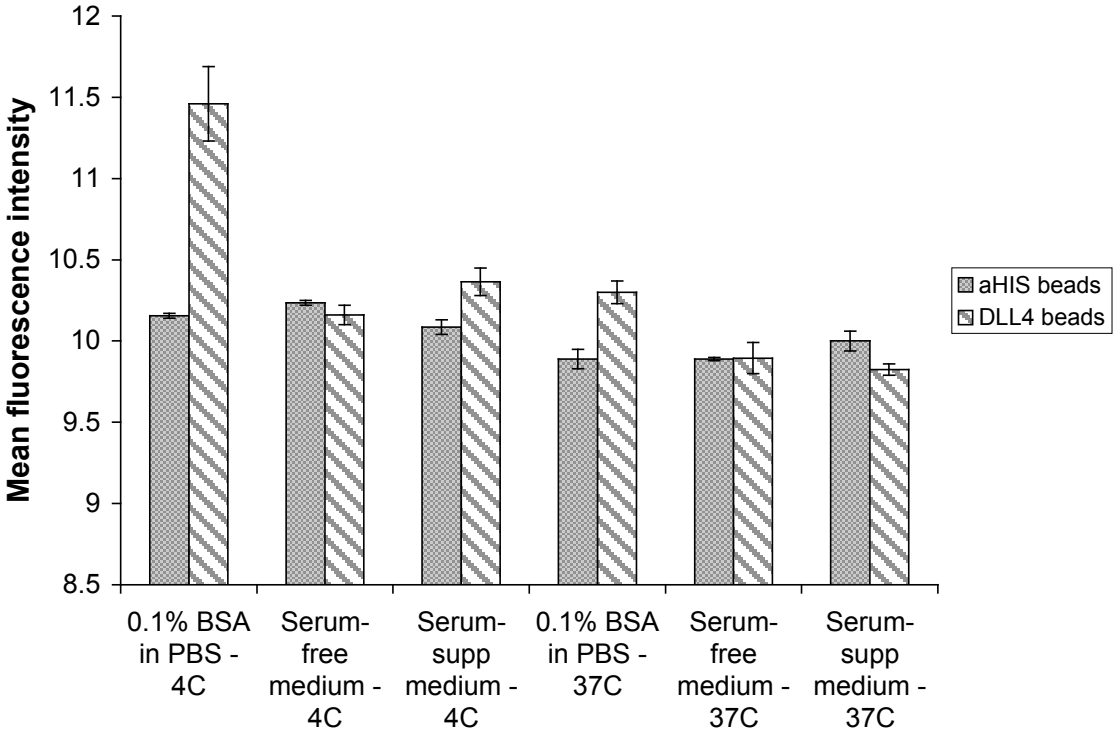


Figure 3.13 Effect of temperature, serum and medium on the stability of DLL4 functionalized Proactive® beads after 1 hr, 1 day and 1 week of incubation. Beads were coated with a predefined amount of anti-6x HIS antibody and 700 ng of DLL4 per million beads and incubated for one hr, one day and one week in PBS, serum-free medium and serum-supplemented medium at 4ºC (for the PBS only) and 37ºC. Following incubation, beads were stained and analyzed for surface DLL4 using flow cytometry analysis. Beads were blocked in 3% BSA in PBS and stained with anti-DLL4 antibody and FITC anti-rat IgG antibody. Average mean fluorescence intensity values are given below with bars indicating standard error. Anti-6x HIS antibody coated beads served as negative controls.

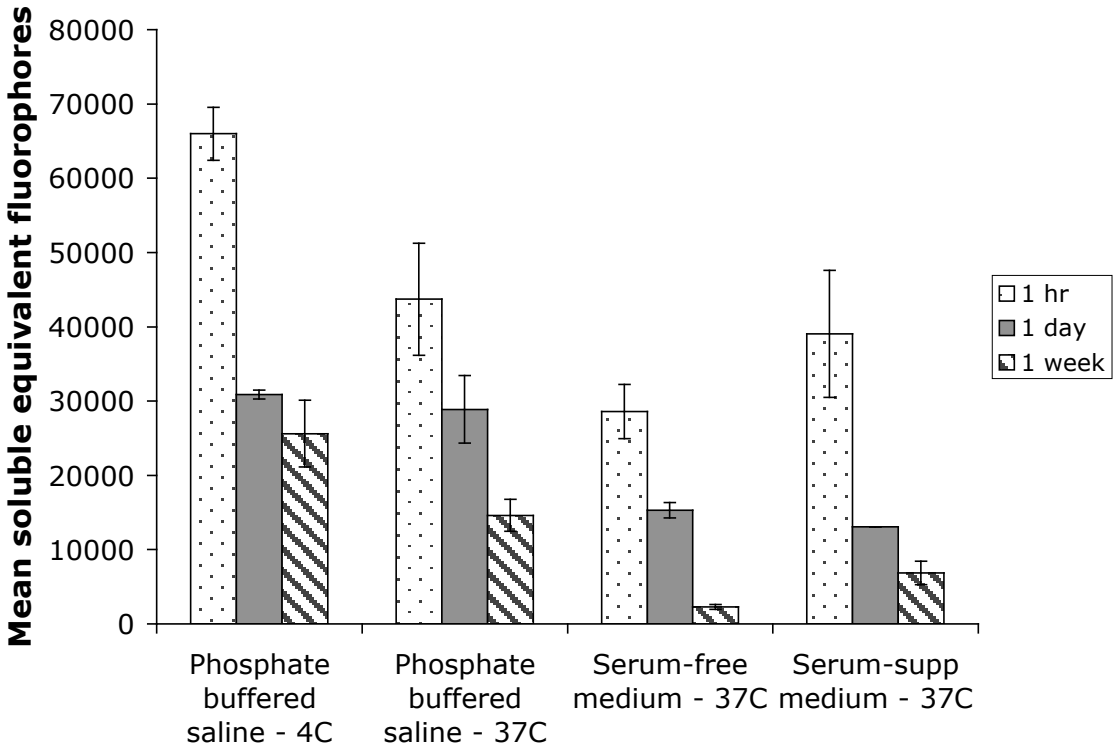


Figure 3.14 Cytotoxicity effects of uncoated and DLL4 functionalized Biotin Binder Kit beads using MTT assay in C2C12 myoblasts. DLL4 functionalized and uncoated beads were added to seeded C2C12 myoblasts at bead to cell ratios of 0.1:1, 1:1 and 5:1 and incubated for 3 days, 7 days and 9 days with cells. After each incubation, a MTT solution was added and incubated with cells for 4 hrs. Formazan crystals were solubilized and solutions measured at 570 nm to assess viability of cells. All values were normalized to cells only condition.

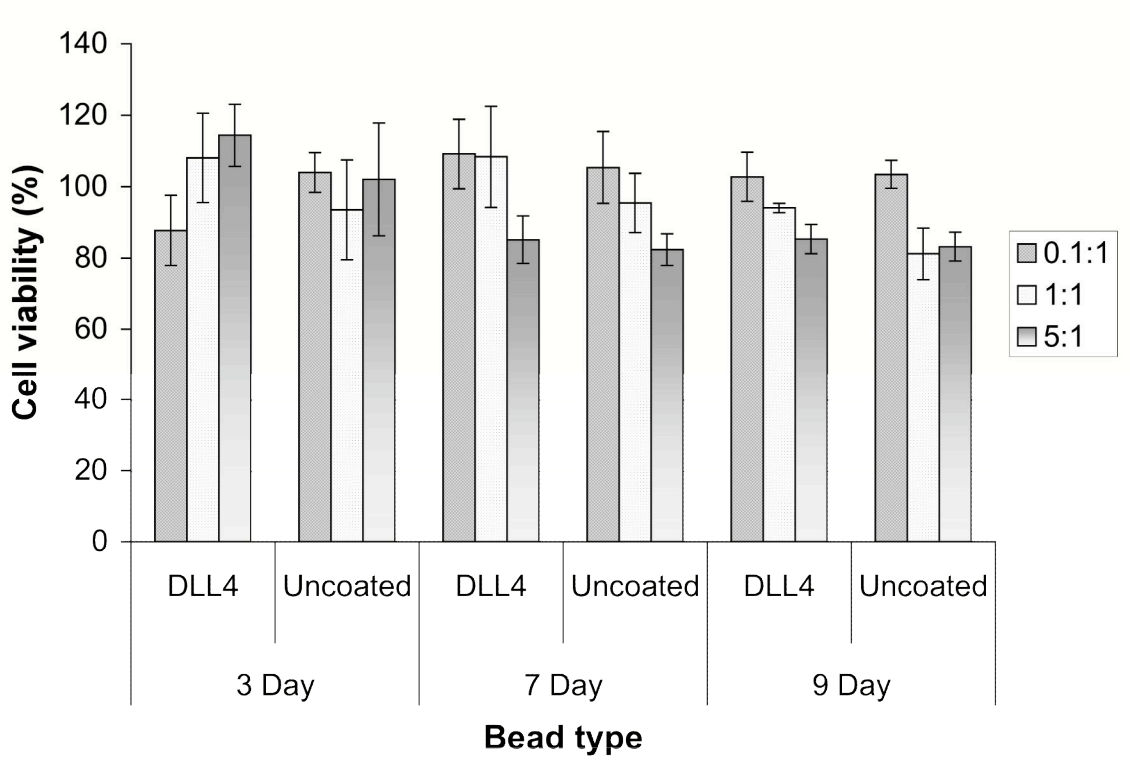


Figure 3.15 Cytotoxicity effects of uncoated and DLL4 functionalized Biotin Binder Kit beads using MTT assay in R1 embryonic stem cells. DLL4 functionalized and uncoated beads were added to seeded R1 embryonic stem cells at bead to cell ratios of 0.1:1, 1:1 and 5:1 and incubated for 3 days, 7 days and 9 days with cells. After each incubation, a MTT solution was added and incubated with cells for 4 hrs. Formazan crystals were solubilized and solutions measured at 570 nm to assess viability of cells. All values were normalized to cells only condition.

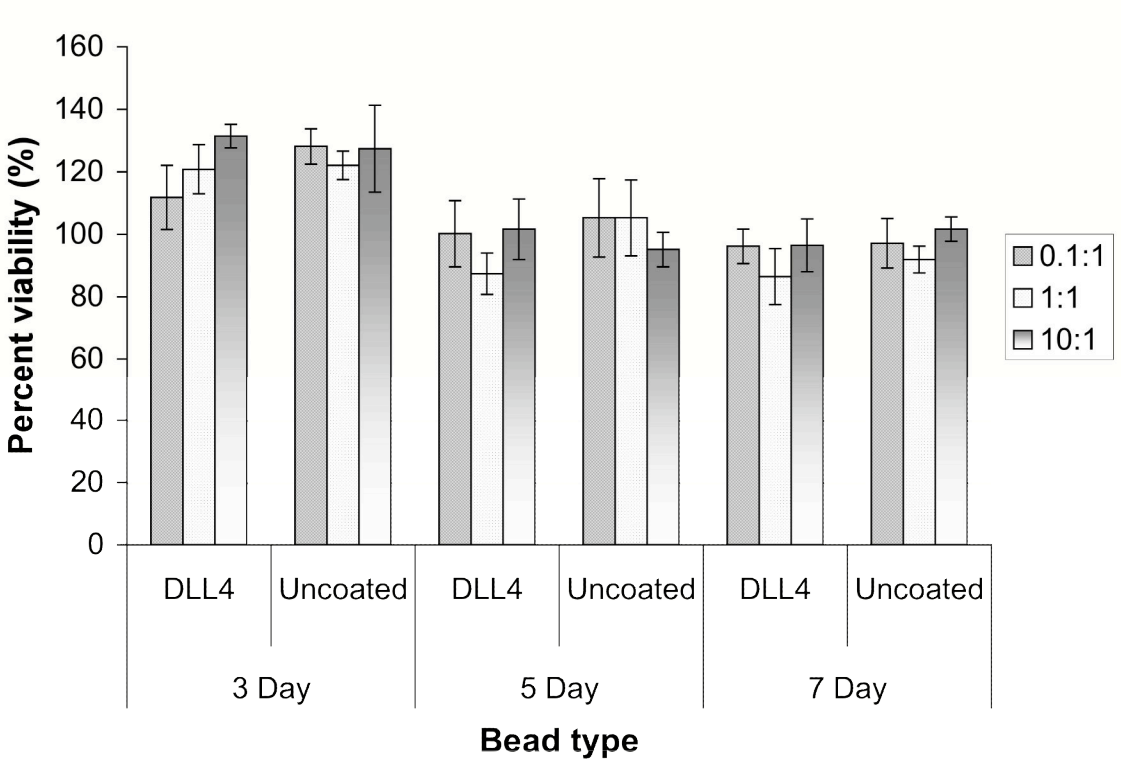


Figure 3.16 Cytotoxicity effects of uncoated Biotin Binder Kit beads using MTT assay in C2C12 myoblasts. Uncoated beads were added to seeded C2C12 myoblasts at bead to cell ratios of 0.1:1, 1:1, 10:1, 20:1 and 40:1 and incubated for 3 days, 5 days and 7 days with cells. After each incubation, a MTT solution was added and incubated with cells for 4 hrs. Formazan crystals were solubilized and solutions measured at 570 nm to assess viability of cells. All values were normalized to cells only condition.

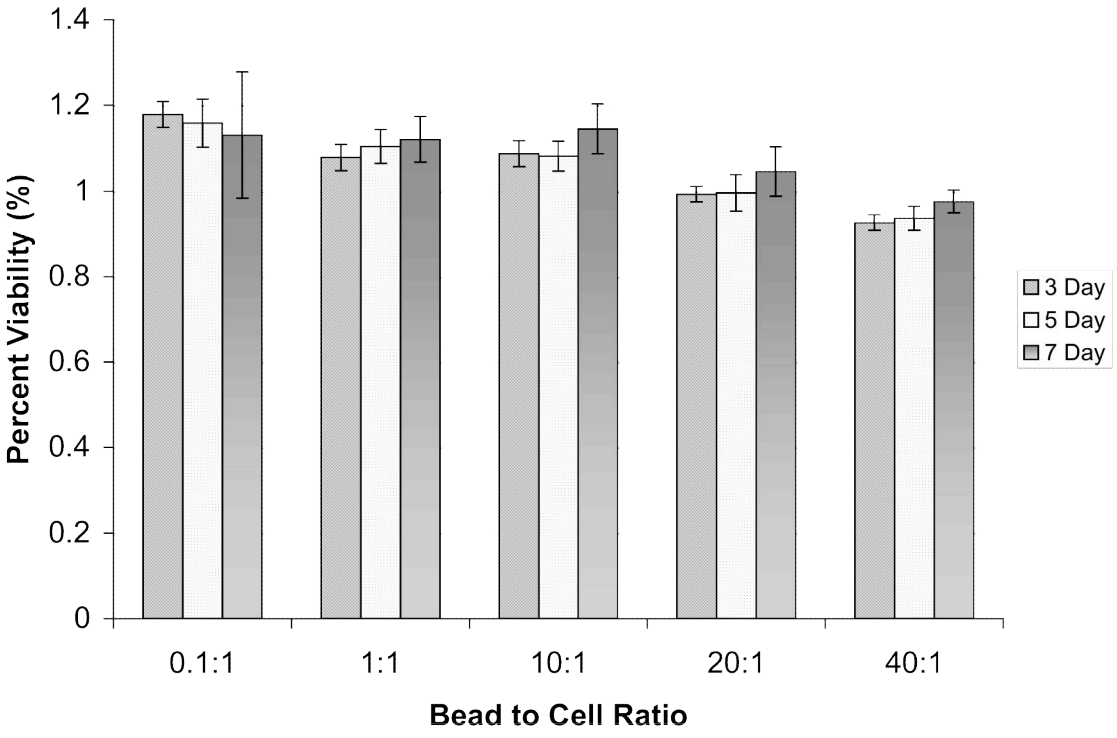
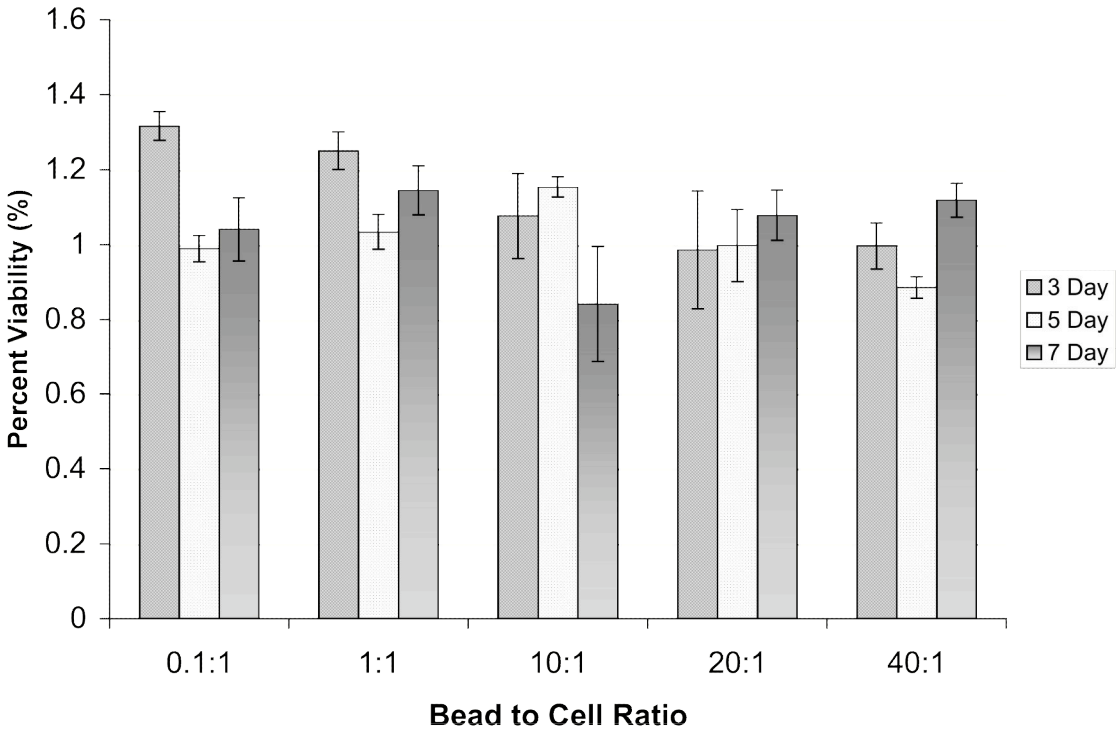


Figure 3.17 Cytotoxicity effects of uncoated Biotin Binder Kit beads using MTT assay in R1 embryonic stem cells. Uncoated beads were added to seeded R1 embryonic stem cells at bead to cell ratios of 0.1:1, 1:1, 10:1, 20:1 and 40:1 and incubated for 3 days, 5 days and 7 days with cells. After each incubation, a MTT solution was added and incubated with cells for 4 hrs. Formazan crystals were solubilized and solutions measured at 570 nm to assess viability of cells. All values were normalized to cells only condition.



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CHAPTER FOUR

Qualitative and Quantitative Assessment of Dose-Dependent Notch Signaling

4.1 INTRODUCTION

We have previously characterized and optimized the fabrication of Notch functionalized microbeads, both with and without a DNA linker, as described in Chapter 3. The use of a bead system boasts two levels of control on the final ligand density presentation, concentration of DLL4 density on bead surface and bead to cell ratio. Previous results have demonstrated low microbead cytotoxicity and stability in serum-supplemented medium for up to a week in the absence of a DNA linker. Our goal is to utilize Notch functionalized beads for the *ex vivo* generation of T cells for adoptive transfer applications. We are also interested in studying the effects, if any, of varying both the ligand density and bead to cell ratios with Notch signaling responsive cells. We hypothesize that such a quantitatively controllable system can be tailored to optimize the amount of Notch signaling by utilizing such a system.

Recent studies have explored the effects of ligand density on T cell differentiation utilizing two main approaches, administering varying amounts of enzyme inhibitor to prevent Notch signaling at the receptor level and immobilizing varying densities of Notch ligands on the surface of polystyrene (Beckstead et al., 2006; Ciofani et al., 2004; Dallas et al., 2005; Delaney et al., 2005; De Smedt et al., 2005; Lehar et al., 2005; Schmitt et al., 2004). The first approach involves the use of OP9-DL1 stromal cell line, which is transfected to express Notch ligand Delta1 or other Notch signaling supportive microenvironments. Previous studies and reviews have explored at length the benefits of

utilizing a Notch ligand transfected stromal cell line in T cell differentiation (de Pooter et al., 2007; Schmitt et al., 2004; Zuniga-Pflucker et al., 2004). As described in Chapter 2, upon Notch ligand-receptor binding, the receptor is cleaved three times prior to the translocation of the intracellular Notch into the nucleus and subsequent expression of Notch target genes. One of the cleavages involves the activity of a presenilin-dependent γ secretase that is responsible for releasing the intracellular portion of Notch for nuclear translocation (Saxena et al., 2001). Two inhibitors of such enzyme activity, presenilin inhibitor X and 7 (N-[N-(3,5-difluorophenyl)-L-alanyl]-S-phenyl-glycine t-butyl ester] DAPT have been readily used to explore the requirements of Notch signaling for developing thymocytes (De Smedt et al., 2005; Lehar et al., 2005; Schmitt et al., 2004). For example, Schmitt and colleagues used varying concentrations of inhibitor X to demonstrate lymphocyte potential in fetal liver-derived HPCs. Lower concentrations of the inhibitor resulted in a greater induction of NK cell development while high concentrations resulted in B cell development and loss of T cell lineage, suggesting a threshold for Notch signaling with T cells having the maximum requirement (Schmitt et al., 2004). Other studies have utilized FTOC culture and OP9 transfected Jagged 1 cell line to explore the Notch signaling thresholds in T cell development (De Smedt et al., 2005; Lehar et al., 2005). De Smedt and colleagues confirmed the threshold requirement for lymphocyte development in human precursor cells using FTOC culture, with the order being B cells, NK cells and T cells with increasing levels of Notch required (De Smedt et al., 2005). Finally, Lehar and colleagues also demonstrated B cell development with high levels of inhibitor X using OP9-DL1 and developing thymocytes. Inhibitor addition, however, did not affect the T cell development for OP9 cells transfected with Notch ligand Jagged 1 (Lehar et al., 2005).

As described above, another approach to quantitatively varying the degree of Notch signaling involves the immobilization of varying amounts of Notch ligand on the surface of polystyrene, therefore directly controlling the amount of Notch ligand present. Similar dose-dependent behavior of developing T lymphocytes has been observed for both murine and human cells using immobilized Notch ligands (Dallas et al., 2005; Delaney et al., 2005). Dallas et al observed marked induction of T cell development for concentrations of 2.5 μg/ml of Delta-1 or higher with little to no B cell commitment as evident from immunophenotype analysis (Dallas et al., 2005). In a related study, Delaney and colleagues observed higher CD7+ expression, an early marker of human T cells, and greater human engraftment at lower densities of immobilized Delta1 (Delaney et al., 2005).

These studies suggest a dose-dependent role of Notch signaling in T cell development and warrant further investigation. Here, we have investigated the effect of these quantitative differences using several qualitative and quantitative assessments, as summarized in **Figure 4.1**. Myotube inhibition has been used to qualitatively demonstrate effective Notch signaling using our bead system. Furthermore, we found basal levels of Notch signaling using both intracellular Notch staining and RTPCR studies in both C2C12 myoblasts and R1 embryonic stem cells. Despite the high background, real-time RTPCR and luciferase results imply a threshold response of myoblasts with increased Notch target gene expression for lower bead to cell ratios and higher luciferase activity for increased ligand densities. Findings such as these illustrate the importance of ligand density, aid in our understanding of Notch signaling and can be further applied to T cell development for the eventual production of therapeutic T cell lymphocytes.

4.2 MATERIALS AND METHODS

4.2.1 Bead fabrication

DLL4 functionalized beads were prepared as described in Chapter 3. Both Biotin Binder Kit (Dynalbiotech, Brown Deer, WI) and Proactive® (Bangs Labs, Fishers, IN) were used for Notch signaling studies.

4.2.2 C2C12 cell and R1 cell culture

R1 embryonic stem cells and C2C12 myoblasts were cultured as described in Chapter 3. Additionally, medium was changed for C2C12 myoblasts after 24 hr of seeding for all studies and differentiation medium was added. Differentiation medium consisted of DMEM (Invitrogen, Carlsbad, CA), 10% equine serum (Hyclone, Logan, UT) and antibiotics. Differentiation medium is known to promote myoblast differentiation and myotube formation.

4.2.3 C2C12 myotube inhibition assay for Notch signaling

Notch signaling has been extensively shown to inhibit the formation of myotubes in C2C12 cells. Thus, myotube inhibition has been used extensively as an assay to demonstrate efficient Notch signaling (Kopan et al., 1994; Lindsell et al., 1995; Luo et al., 1997; Varnum-Finney et al., 2000). Myoblast differentiation was conducted similarly to the methods described by Varnum-Finney et al (Varnum-Finney et al., 2000). First, concentrations of DLL4 (R&D Systems, Minneapolis, MN) were immobilized and tested for C2C12 differentiation. DLL4 was immobilized at concentrations of 1 μg/ml and 1.5 μg/ml onto a 96 well plate for 2-3 hours. After immobilization, wells were washed three times with PBS and seeded with C2C12 (ATCC, Manassas, VA) at a concentration of

200000 cells/ml in C2C12 maintenance medium. Cells without DLL4 were used as the negative control. Medium was changed after one day and replaced with differentiation medium. Qualitative analysis of myoblast differentiation and Notch activation was conducted using visualization of myofibril formation, as described before (Varnum-Finney et al., 2000). Next, experiments were performed where the bioactivity of soluble DLL4 was assessed. In these studies, DLL4 was immobilized as described above at a concentration of 1 μg/ml. Soluble DLL4 was added to additional wells at 1 μg/ml and cells without DLL4 were included as the negative control. Medium was replaced as described above and soluble DLL4 was replaced for relevant wells. Imaging was performed after 6 days of incubation using phase contrast microscopy. To ensure controlled orientation of DLL4, a directional binding scheme was utilized in bead fabrication. This binding scheme consists of streptavidin coated beads with biotinylated anti-6x HIS antibody and histidine tagged DLL4. Supplemental studies were conducted where each of the components of the binding scheme were immobilized and tested in terms of myoblast differentiation using methods as described above. For all protein immobilization conditions, proteins were coated for 2 hours at 2 μg/ml at 37°C. Proteins included neutravidin (Pierce Biotechnology, Rockford, IL), biotinylated anti-6x HIS antibody (R&D Systems, Minneapolis, MN) and recombinant DLL4 (R&D Systems, Minneapolis, MN). For neutravidin-biotinylated anti-6x HIS-DLL4 condition, wells were blocked with 1% BSA at 37°C following the antibody incubation. Finally, DLL4 coated beads were also incubated with C2C12 cells and tested for myotube inhibition as described above. Cells were seeded one day prior to bead addition. Ligand coated beads were added to wells at bead to cell ratios of 1:1 and 5:1. Uncoated Biotin Binder kit beads (Dynalbiotech, Brown Deer, WI), added at the same bead to cell ratios, served as controls. All conditions were performed in triplicate. Cells were visualized using the

20x objective of the Leica fluorescent IRB inverted microscope (Meyer Instruments, San Antonio, TX).

4.2.4 Intracellular Notch staining

To demonstrate effective Notch signaling, myoblasts and stem cells were incubated with Notch functionalized beads and stained for activated Notch. R1 cells and C2C12 cells were seeded at a concentration of 20000 cells and 75000 cells, respectively, per poly-L-lysine coated coverslips one day prior to bead incubation. Cells were then fixed in 4% paraformaldehyde, washed with phosphate buffered saline (PBS), and then permeabilized with 0.25% Triton X-100 for 10 minutes are room temperature and subsequently washed one more time. Samples were blocked in 1.5% goat serum in PBS for 1 hour, and antibodies were diluted 1:750 with 1% BSA, 0.05% sodium azide in PBST. Cells were incubated overnight at 4°C with primary antibody rabbit anti-mouse/human activated Notch antibody (Abcam, Cambridge, MA), incubated 1 hour at room temperature with Alexafluor 488 goat anti-rabbit antibody (Invitrogen, Carlsbad, CA), washed with PBST, and stained with DAPI (Invitrogen, Carlsbad, CA) at a concentration of 300nM for nuclear visualization. Finally, coverslips were mounted with Permount mounting medium (Fisher Scientific, Pittsburg, PA), sealed with clear nail polish and stored in the dark at 4ºC. Cells were imaged with the Zeiss Apotome Axiovert 200 microscope (Carl Zeiss Microimaging, Thornwood, NY). Additional studies were also conducted where the addition of Notch functionalized beads and uncoated beads were performed. The degree of intracellular Notch activation was assessed using staining as described above with anti-human/mouse ICN antibody-FITC (eBioscience, San Diego, CA). After staining, cells were washed and resuspended in 250 μl of staining buffer and

analyzed using FACSCalibur (Becton Dickinson, San Diego, CA) and CellQuest 3.1 software (BD Biosciences, San Jose, CA).

4.2.5 Real-time RT-PCR studies of Notch gene expression

Cytoplasmic RNA was isolated from cells using the methods described previously (Gore and Roberts 1993). Briefly, frozen cell pellets were first separated into nuclear and cytoplasmic fractions using a lysis buffer. Protein was removed from the cytoplasmic fraction using proteinase-K (200 pg/ml final concentration; Roche Applied Science, Indianapolis, IN) at 45ºC. Next, a phenol-chloroform-isoamyl alcohol extraction at ratios of 25:24:1 was performed followed by an additional chloroform extraction and isopropanol precipitation at -20ºC. After washing fractions with 70% ethanol, pellets were dried and resuspended in nuclease-free water. Quality of isolated RNA was assessed using ND-1000 spectrophotometer (Nanodrop, Wilmington, DE). Contaminating DNA was removed from pellets using TURBO DNA-free kit (Ambion, Austin, TX) according to manufacturer's instructions. Complementary DNA was synthesized using the Superscript First-Strand Synthesis Kit (Invitrogen, Carlsbad, CA) using at least 500 ng of RNA. Reaction ready SYBR® Green qPCR mix (Superarray, Frederick, MD) was used to perform quantitative polymerase chain reaction (PCR) on ABI Prism 7900 HT Sequence Detector (Applied Biosystems, Foster City, CA). Amount of gene expression was deduced from threshold cycle values. For RT-PCR studies, DNA was amplified from mRNA with AccessQuick™ Master Mix (2X) and AMV reverse transcriptase (Promega, Madison, WI) as per manufacturer's instructions. PCR consisted of 40 cycles of 45 s at 94°C, 1 min at 60°C, and 1 min at 72°C. PCR products were separated by 2% agarose gel electrophoresis and were visualized with ethidium bromide staining. Gene specific primers for HES1, Hrp2, NRARP, Beta actin and GAPDH were

all acquired from Superarray. Beta actin and GAPDH were used as housekeeping genes to normalize data.

4.2.6 Luciferase reporter assay

C2C12 cells were seeded onto 24 well plates to achieve 70% confluence after 24 hours. Cells were transfected with 4xwtCBF1Luc plasmid (gift from D. Hayward, Johns Hopkins University, Baltimore, MD) at 1 μg/ml using Exgen 500 (Fermentas, Glen Burnie, MD) according to manufacturer's instructions. Plasmid was amplified at Aldevron (Aldevron LLC, Fargo, ND). After 24 hours of transfection, DLL4 functionalized and uncoated beads were added to cells in serum-free differentiation medium at designated bead to cell ratios. Cells were washed and lysed after 24 hours using Glo Lysis Buffer (Promega, Madison, WI). Protein content in samples was assessed using Micro BCA Protein Assay Kit (Pierce, Biotechnology, Rockford, IL), as per manufacturer's protocol. Lysate was evaluated for luciferase expression using a Dynex MLX Luminometer (Dynex, Chantilly, VA). RLU values were normalized to protein content using BCA results. All conditions were performed with at least n=3.

4.3 RESULTS

4.3.1 Immobilization of DLL4 is necessary for Notch signaling; functionalization scheme does not affect myotube inhibition

The inhibition of myotube formation is a classical assay performed to demonstrate efficient signaling through Notch ligands. It has been demonstrated that the presence of immobilized Notch ligands inhibits the spontaneous differentiation of C2C12 myoblast cells in myotubes (Varnum-Finney et al., 2000). To evaluate the bioactivity of

immobilized DLL4, we performed a C2C12 myoblast differentiation assay and monitored the degree of myotube inhibition. First, we immobilized various concentrations of DLL4 onto a polystyrene 96 well plate and assessed the degree of myotube inhibition after 5-6 days of incubation using phase contrast microscopy. As shown in **Figure 4.2**, myotube inhibition occurred at both concentrations of immobilized DLL4 tested, 1 μg/ml and 1.5 μg/ml, to similar degrees. In contrast, myotube formation was observed for wells without DLL4. Next, we evaluated the need for immobilization by adding DLL4 at 1 μg/ml in the immobilized and soluble forms to myoblasts and observing myotube formation. Myotube formation was inhibited to a small extent with soluble DLL4 but nevertheless resulted in myotube formation after the 5-6 day incubation. The myoblast morphology is retained only when DLL4 was immobilized, as shown in **Figure 4.3**. Finally, to ensure the functionalization scheme did not affect myotube formation or in fact have an inhibitive effect, neutravidin, biotinylated anti-6x HIS antibody and the functionalization scheme (neutravidin-biotinylated anti-6x HIS antibody-HIS tagged DLL4) were all evaluated. Proteins were immobilized at 2 μg/ml, extensively washed and seeded with myoblasts. **Figure 4.4** shows the presence of myotubes in all conditions except those where DLL4 was immobilized after 6 days of incubation. The functionalization scheme does not appear to have any additional effect, as evident by the similar morphology of cells in **Figure 4.4d and Figure 4.4e**.

4.3.2 DLL4 functionalized microbeads can provide efficient Notch signaling

The degree of myotube inhibition in the presence of DLL4 functionalized microbeads was qualitatively evaluated through phase contrast microscopy after 6 days of culture. Streptavidin coated beads were used as negative controls. **Figure 4.5** indicates that the presence of Notch ligand on the microbead surface significantly inhibits myotube

length and maturation. (Myotubes are indicated by red arrows.) Although the presence of some myotube formation is suggested by the elongated morphology of the myoblasts in **Figures 4.5c and 4.5d**, the mature myotube morphology is markedly reduced as compared to **Figures 4.5a and 4.5b** (all images in **Figure 4.5** are of 20x magnification). Increasing the functionalized bead to cell ratio from 1:1 to 5:1 had no visible effect in terms of increased myotube inhibition or retention of myoblast morphology, as evident in **Figures 4.5c and 4.5d** indicating a saturation effect. This data suggests a bead to cell ratio of 1:1 is sufficient to transmit Notch signals and significantly inhibit myotube formation in the C2C12 myoblast system.

4.3.3 Basal levels of intracellular Notch staining exist in R1 embryonic stem cells and C2C12 myoblasts

In the previous section, we demonstrated that immobilized DLL4 (both on polystyrene and microbeads) could provide Notch signaling to C2C12 myoblasts as evident by the cell morphology and level of differentiation. Here, we wished to further investigate the level of Notch signaling on a more molecular level. Notch signaling is known to induce a cleavage in the ligand bound Notch receptor releasing an intracellular Notch (ICN) portion of the Notch receptor (Maillard et al., 2005). The ICN can be stained in fixed and permeabilized cells and used to assess the degree of Notch signaling. We first assessed the basal level of Notch signaling in two types of cells, myoblasts and embryonic stem cells. Cells were stained for the presence of intracellular Notch antibody and imaged for a qualitative analysis. SK-N-MC neuroepithelioma cells are known to always express Notch and served as positive controls (Ohishi et al., 2002). As shown in **Figure 4.6b**, a significant amount of Notch signaling without any prior Notch ligand addition existed in myoblasts. Embryonic stem cells also displayed similar levels of

basal Notch signaling as shown in **Figure 4.7b**. In all images acquired, the ICN were localized in the nuclei for both myoblasts and stem cells, as shown in **Figure 4.6b and Figure 4.7b.** Additional studies were conducted where Notch functionalized beads were incubated with myoblasts and cells were assessed for Notch signaling using ICN staining and flow cytometry analysis (data not shown). These studies were conducted on cells with suspension, attached and pellet culture for varying incubation periods (1hr, 1 day, 1 wk) with little to no differences between conditions with and without Notch functionalized beads. Proactive beads® were also functionalized and tested for intracellular Notch activation with similar results. Differences in Notch signaling, at least on the level of activated ICN, were most likely minimal and difficult to detect using this assay due to the high background levels.

4.3.4 Real-time and end point RTPCR analysis indicate upregulation of Notch target genes in myoblasts

To investigate Notch signaling at a more sensitive level, we performed RTPCR studies using both end point and real-time quantitative analyses. First, RNA was isolated from R1 embryonic stem cells for a week and evaluated for inherent Notch signaling, using a Notch target gene. RNA was isolated on days 1, 3, 5 and 7, treated for DNA contamination, converted to cDNA and amplified using HES1 and beta actin specific primers. Amplified DNA was then run on agarose gels and stained using ethidium bromide. **Figure 4.8** shows basal levels of HES1 expression even after 1 week of culture. Real-time RTPCR studies were also performed on R1 ESCs incubated with Notch functionalized beads at varying bead to cell ratios. These results revealed no difference when comparing both uncoated and Notch functionalized beads and bead to cell ratios (data not shown).

Next, we examined Notch gene upregulation in myoblasts with both Notch functionalized Biotin Binder Kit and Proactive® beads. First, we added Notch functionalized Biotin Binder Kit microbeads and uncoated beads at bead to cell ratios of 0.1:1 and 1:1 and studied the upregulation in HES1 gene expression. HES1 and HERP2 genes are known to be common Notch target genes in myoblasts (Iso et al., 2001). All values were normalized to cells only expression levels. **Figure 4.9** shows the 5- and 4-fold increase in gene expression for the 0.1:1 and 1:1 bead to cell ratios when comparing DLL4 and uncoated beads, respectively, after 2 days of bead-cell incubation. Additional incubation where beads and cells were incubated for 6 days exhibited similar results, as shown in **Figure 4.10**. Notch functionalized Proactive® beads had a similar effect on Notch target gene upregulation. Here, beads were added at bead to cell ratio of 0.5 to 1 and HES1 and HERP2 target genes were studied. **Figure 4.11** shows a 3-fold and 2-fold change in gene expression with the addition of 1200 ng of DLL4/million bead and 2000 ng of DLL4/million bead ligand densities, respectively over anti-6x HIS antibody beads. Student's *t* test indicated a p value of 0.08 when comparing the 1200 ng of DLL4/million bead ligand density to anti-6x HIS antibody bead for the HERP2 gene.

4.3.5 Luciferase reporter assay indicates increase in Notch related CBF-1 activity in a dose dependent manner in myoblasts with Notch functionalized beads

In mammalian systems, upon Notch ligand binding, there are a series of cleavages in the Notch receptor one of which results in the release of the intracellular domain (ICN) and translocation to the nucleus, as described above. Upon entry into the nucleus, ICN associates with the transcription factor CBF1/RBP-J and initiates activation of Notch target genes (Maillard et al., 2005). Here, we utilized a CBF1-Luc reporter to demonstrate the dose-dependent effects of DLL4 functionalized beads on Notch

signaling, directly. The CBF1-Luc reporter has CBF1 binding elements cloned into a GL2pro plasmid (Hayward et al., 1996) and can be used to demonstrate Notch activity based on luciferase gene expression. First, we tested the DLL4 functionalized Biotin Binder Kit beads for a dose dependent response in C2C12 cells using the luciferase reporter assay. Transfected myoblasts were incubated with functionalized and uncoated beads at bead to cell ratios of 1 to 1 and 5 to 1 for 24 hr and evaluated for luciferase expression. Results indicated an increase in luciferase expression, as shown in **Figure 4.12**, for both 1:1 and 5:1 bead to cell ratios, when comparing DLL4 functionalized and uncoated beads. Student's *t* test showed a statistically significant difference for both ($p<0.05$). When comparing the luciferase expression of the DLL4 functionalized beads to the cells only, however, no statistical difference was found for both 1:1 and 5:1 (p values of 0.25 and 0.06, respectively).

DLL4 functionalized Proactive® beads were also incubated with transfected myoblasts and tested for their effects on luciferase expression. Here, however, beads were functionalized with varying amounts of DLL4 ligand densities, to further investigate the dose-dependent response, if any. Beads were functionalized with low, medium and high amounts of DLL4 (200, 600, 2000 ng of DLL4/ million beads) and incubated with transfected myoblasts at various bead to cell ratios including 0.5:1, 1:1, 2:1 and 5:1. Anti-6x HIS coated beads were also incubated with cells at the bead to cell ratios, given above. Cells were then lysed following 24 hours of incubation and tested for luciferase activity. As shown in **Figure 4.13 and Table 4.1**, no clear trend was detected for increasing bead to cell ratios with few statistically significant differences found. When comparing bead to cell ratios for each density with the appropriate anti-6x HIS coated bead to cell ratio, a statistically significant difference was found for all bead to cell ratios for the highest ligand density except 2:1. Comparisons with cells only values also gave

statistically significant differences (**Table 4.2**). Low and medium ligand density beads, however, did not show an upregulation in luciferase activity in a statistically significant manner, with the exception of 0.5:1 bead to cell ratio for the medium ligand density. **Table 4.3** summarizes the fold differences when comparing the highest ligand density coated beads to both anti-6x HIS coated beads and cells. The 5:1 bead to cell ratio resulted in the greatest induction of luciferase activity with a 2.35-fold increase over cells. Additional studies demonstrated up to a 3-fold increase in luciferase activity for the 5:1 bead to cell ratio (with high ligand density) over anti-6x HIS coated beads. In these studies, however, cells without beads had high luciferase activity resulting in only a 1.41-fold increase for the same condition (data not shown).

4.4 DISCUSSION

Notch ligands have been established as some of the key players in T cell differentiation (Ciofani and Zuniga-Pflucker 2007; Heinzel et al., 2007; Maillard et al., 2005; Parreira et al., 2003; Petrie and Zuniga-Pflucker 2007). Notch ligands are known to be present in the thymus on supportive stroma. Recent studies have shown varying densities of Notch ligands in the thymic microenvironment, unique to the signals required for developing thymocytes in each niche. For example, both Delta1 and Delta4 ligands are expressed at a high density in the cortico-medullary junction, the site of entry for thymic progenitors (Heinzel et al., 2007; Schmitt et al., 2004). Although irreversible T cell commitment does not take place here, a high density of Notch ligands ensures T lineage specification and prevents commitment to B cell and NK cell lineages for example (Petrie and Zuniga-Pflucker 2007). The medulla, known as the site of positive selection and exportation, on the other hand, boasts a very low density of Delta ligands (Heinzel et al., 2007). Developing thymocytes are nearly mature once they reach the

medulla and most likely do not need Notch signals for functional maturation (Petrie and Zuniga-Pflucker 2007).

This idea of density-dependent Notch signaling and T cell development has recently been explored and has led to a better understanding of developmental potential and necessary levels of Notch signaling (Beckstead et al., 2006; Ciofani et al., 2004; Dallas et al., 2005; Delaney et al., 2005; De Smedt et al., 2005; Lehar et al., 2005; Schmitt et al., 2004). Use of γ secretase inhibitors such as presenilin inhibitor X and DAPT in Notch ligand supportive stroma suggest a Notch signaling threshold capable of committing progenitors at varying levels of Notch ligand density (Ciofani et al. 2005; De Smedt et al., 2005; Lehar et al., 2005; Schmitt et al., 2004). The current hypothesis suggests that T cells, NK cells and B cells require increasing levels of Notch signaling, with T cells requiring the greatest level and B cell requiring the least. Moreover, providing a high degree of Notch signaling obligates T cell specification and prevention of B cell commitment. One disadvantage of such an indirect method of controlling levels of Notch signaling, however, is the failure to quantitatively characterize the Notch signaling needed.

A more direct method of "measuring" Notch signaling has recently been studied where varying concentrations of Notch ligands are immobilized on the surface of polystyrene (Dallas et al., 2005; Delaney et al., 2005). Dallas and colleagues observed significant induction of T cell commitment in murine progenitors at concentrations of 2.5 μg/ml of DLL1 or greater. These studies also confirmed the presence of little to no B cell commitment at such concentrations. Similar results were observed for human progenitors, as well (Delaney et al., 2005).

Both methods have made significant contributions to our understanding in T cell development and represent two examples of *ex vivo* differentiation schemes. Coculture

studies with Notch ligand transfected cell stroma more closely represent the thymic microenvironment but suffer from the inability to quantify the amount of Notch signaling and provide a cell-free microenvironment, both of which are important for scaling up the production of human T cells. Immobilized Notch ligand is better suited for scaling up T cell generation in terms of a cell-dependent system but may pose expense issues. Also, in both methods, the removal of Notch signaling from the culture environment for possible optimization or developmental studies can be difficult to achieve.

We have addressed such limitations with the functionalization of Notch ligands on commercially available microbeads. Our design exploits the highly directional and high strength binding of streptavidin-biotin and antigen-antibody interactions to provide Notch ligands in a quantifiable manner. Collectively, there are two levels of ligand density control in this design, ligand density on each bead and bead to cell ratio. In this study we have, we have extensively studied the effect of bead to cell ratio using several qualitative and quantitative assays at both the extracellular and intracellular levels. These assays are summarized in **Figure 4.1**.

We first used the myotube inhibition assay to demonstrate Notch signaling at the level of cellular morphology. Notch signaling has been extensively shown to inhibit myotube formation and maintain the myoblast phenotype through primarily coculture studies (Iso et al., 2001; Kopan et al., 1994; Kuroda et al., 1999; Lindsell et al., 1995; Luo et al., 1997; Mizutani et al., 2000; Nofziger et al., 1999; Varnum-Finney et al., 2000). In these studies, myeloma, fibroblast and fibrosarcoma cell lines were transfected to express Notch ligands such as Jagged 1 or Delta 1 and used to characterize the signal transduction and gene expression responses to Notch signaling in C2C12 cells (Iso et al., 2001; Kuroda et al., 1999; Mizutani et al., 2000; Nofziger et al., 1999). These studies also suffer from the inherent dependence on a transfected cell line to present the ligand.

Varnum-Finney and colleagues have utilized another approach in C2C12 cells, namely ligand immobilization (onto polystyrene), and have elegantly demonstrated Notch signaling and myotube inhibition only when Notch ligand Delta 1 is immobilized. Significant reduction of a selected myotube marker (myosin) could be observed in these studies with 1 μg/ml of adsorbed Delta 1 after 2-3 days of differentiation (Varnum-Finney et al., 2000).

DLL4 is a member of the Delta family of Notch ligands and has been extensively studied in T cell and vasculature applications. To demonstrate effective Notch signaling, studies were first conducted with increasing amounts of plate adsorbed Notch ligand and C2C12 differentiation was monitored. These studies indicated myotube inhibition after 5-6 days with 1 μg/ml of Delta 4, which is consistent to previous plate adsorbed Delta 1 studies (Varnum-Finney et al., 2000). Immobilization of Notch ligand Delta 1 has been shown to be necessary for myotube inhibition while non-immobilized forms thwarted Notch activation. We conducted a similar assay here where Delta 4 was cultured with C2C12 cells at a concentration of 1 μg/ml in both the immobilized and soluble forms and myotube inhibition studied. Our results were consistent with Delta 1 studies and demonstrated myotube inhibition with soluble forms of Delta 4. Additional studies were also performed to confirm that the myotube inhibition was in fact a result of the Delta 4 ligand and not any other component of the functionalization scheme. Myotube inhibition was only observed in wells where DLL4 was immobilized, implying no inhibitive effect of streptavidin or biotinylated anti-6x HIS antibody. Finally, to verify whether DLL4 functionalized microbeads can effectively provide Notch signaling, we performed the myotube inhibition assay described above. DLL4 functionalized or unmodified beads at various bead to cell ratios were used. We demonstrated that a bead to cell ratio of 1:1 was sufficient to inhibit myotube formation. Although some myotube-like structures

were still evident, the tubes appear to be significantly smaller and less structured compared to cells cultured with uncoated beads, demonstrating myotube inhibition. These studies were performed with Notch ligand functionalized Biotin Binder Kit microbeads, which have been established as having low stability (Chapter 3). Thus, these results seem counterintuitive. We speculate the clustering or aggregation of released DLL4 may lead to the activation of Notch receptors. These results suggest the Notch ligand functionalized microbeads can provide Notch signaling to myoblasts as evident on a phenotypic level.

As described earlier, Notch ligand-receptor binding induces several cleavages of the Notch receptor, one of which results in the release and translocation of intracellular Notch (responsible for activating Notch target genes). Prior to bead addition, intracellular staining revealed basal levels of Notch signaling in both myoblasts and R1 embryonic stem cells. This result is echoed in studies by Delgado et al where the induction of Notch1 and Notch3 were found in differentiating C2C12 myoblasts, suggesting the induction of the Notch pathway (Delgado et al., 2003). Other studies have indicated the presence of Notch ligands, such as Jagged 1, Jagged 2, Delta 1 and Delta 3 ligands in undifferentiated embryonic stem cells suggesting the induction of Notch signaling from cell-cell contact. Despite the basal levels of Notch signaling, myoblasts were stained for intracellular Notch after the addition of varying levels of Notch ligand functionalized beads. Our results indicated no difference in fluorescence levels when comparing conditions with and without Notch beads. We believe that the high background may have masked the differences, if any, in the conditions. Also, the Notch activation complex is degraded rapidly, further complicating the measurement of intracellular Notch signaling (Maillard et al., 2005). Both Biotin Binder Kit and Proactive® beads gave similar results in ICN staining studies.

Next, we examined the upregulation of Notch target genes using RT-PCR analysis in R1 embryonic stem cells and C2C12 myoblasts. HES1, HERP2 (HEY1) and NRARP are all established Notch target genes in embryonic stem cells (Nemir et al., 2006, Schmitt et al., 2004). In our studies, basal levels of Notch target gene HES1 could still be observed in undifferentiated embryonic stem cells after one week of culture demonstrating the expression of Notch ligands and cell-cell contact. These results confirm the observed ICN staining results. Nemir and colleagues found similar results with HES1 and HERP2 expression in both 6 day and differentiated embryoid bodies (Nemir et al., 2006). Furthermore, no difference in Notch target gene expression between uncoated and Notch functionalized beads was observed in studies assessing the effects of Notch functionalized bead to cell ratio (Biotin Binder Kit), implying the high signaling strength of cell surface Notch ligands. In C2C12 myoblasts, however, Notch target gene upregulation was observed. Both Biotin Binder Kit and Proactive® beads were used for these studies. Both HES1 and HERP2 have been readily used as Notch target genes for the myoblast system, although studies suggest stronger (6-fold) and longer lasting induction for HERP2 in C2C12 cells upon coculture with Delta 1 expressing cells (Iso et al., 2001). Notch ligand functionalized Biotin Binder Kit microbeads were incubated with myoblasts for two and six days before RNA isolation and subsequent gene analysis. Both timepoints indicated a marked induction in HES1 gene expression for the low bead to cell ratio, 0.1:1, nearly 5-fold for the real-time RTPCR analysis. Several studies have shown similar results. Dallas and colleagues indicated a 1.4-fold increase in HES1 expression at 2.5 μg/ml and 2.6-fold increase in HES1 expression at 10 μg/ml of immobilized Delta 1 ligand concentrations in murine lin-sca-1+cKit+ progenitors (Dallas et al., 2005). In human CD34+CD38- precursors, studies demonstrated a range of 2.5-fold to 10-fold increase in HES1 expression for immobilized Delta 1 concentrations

varying from 1.25 μg/ml to 5 μg/ml (Delaney et al., 2005; Ohishi et al., 2002). In addition, the fold difference is on the same scale as the nearly 6-fold increase in luciferase activity for HES1 in U20S fibroblast cell line for 10 μg/ml immobilized Delta 1 (Varnum-Finney et al., 2000). Also, the 6-day timepoint results indicate a sustained response to the Notch functionalized beads. Addition of Notch ligand functionalized Proactive® beads at a bead to cell ratio of 0.5:1 with medium ligand density also resulted in a 3-fold increase of HERP2 expression for myoblasts, after 4 hr of incubation. For both beads, higher bead to cell ratios were used but did not result in an observable induction of Notch target genes. This most likely is a result of the inherent limitation of the bead system, bead aggregation. Notch functionalized and uncoated beads alike aggregate at high bead to cell ratios, resulting in lower effective Notch ligand presentation. At low bead to cell ratios, however, aggregation does not occur, displaying the predicted amount of Notch ligand on bead surfaces to the cells (not taking into account any stability problems).

Finally, we conducted another assay at the molecular level where CBF1 promoter activity was monitored using CBF1-luciferase reporter system. Biotin Binder Kit beads and Proactive® beads were both used for this assay. Addition of Notch ligand functionalized Biotin Binder Kit beads resulted in a statistically significant difference when comparing both Notch ligand functionalized and anti-6x HIS antibody coated beads at both 1:1 and 5:1 bead to cell ratios. Differences between Notch beads with cells, however, were not statistically significant, perhaps due to the stability issues of the beads. Notch ligand functionalized Proactive® beads, however, demonstrated a marked induction of luciferase activity when comparing coated and uncoated beads and coated beads with cells. For these studies, we immobilized three different densities of Notch ligand onto the surface of beads and incubated transfected myoblasts with varying ratios

of all three bead types (with anti-6x HIS antibody coated beads and cells). Although no clear trend could be observed in varying bead to cell ratio, a statistically significant difference was found for the highest ligand density when comparing both cells and anti-6x HIS antibody coated beads (to Notch functionalized beads). Our results indicated a 2.35 fold increase in luciferase activity when comparing the highest ligand density with largest bead to cell ratio to luciferase activity from cells. These results are comparable to CBF-1 activation studies in epithelial stem cells with immobilized Jagged 1 ligand. Beckstead and colleagues indicated a 10-15 fold increase in luciferase activity (using a CBF-1 reporter) when culturing epithelial cells with 1 and 10 nM concentrations of Jagged 1 (Beckstead et al., 2006). It is difficult to directly compare our results to other systems due to the aggregation of beads and any differences in signaling due to the bead design (as opposed to immobilized protein, namely differences in ability to cluster Notch receptors). Requirement of the high ligand density observed in our beads can be attributed to increased ability to cluster Notch receptor and thus, result in Notch receptor activation.

In this study, we have successfully illustrated the biological activity of a novel Notch ligand functionalized microbead through the inhibition of C2C12 differentiation, expression of Notch target genes, and upregulation of CBF1 activity. Our results suggest low bead to cell ratios and high DLL4 ligand densities to be the most effective in inducing Notch signaling for the myoblast system most likely due to the lack of bead aggregation and high Notch receptor clustering. These results suggest a threshold response for Notch signaling in myoblasts and require future studies for understanding the ligand density dependent behavior.

Table 4.1 Student's *t* test analysis of differences among bead to cell ratios for varying DLL4 densities on Proactive® beads for luciferase reporter assay

	1:1	**2:1**	**5:1**
Low 200 ng DLL4/million beads			
0.5:1	***<0.05***	>0.05	>0.05
1:1	-----	***<0.05***	***<0.05***
2:1	-----	-----	>0.05
Medium 600 ng DLL4/million beads			
0.5:1	>0.05	>0.05	>0.05
1:1	-----	>0.05	>0.05
2:1	-----	-----	>0.05
High 2000 ng DLL4/million beads			
0.5:1	>0.05	>0.05	>0.05
1:1	-----	>0.05	>0.05
2:1	-----	-----	***<0.05***
Anti-6x HIS antibody beads			
0.5:1	>0.05	>0.05	***<0.05***
1:1	-----	>0.05	>0.05
2:1	-----	-----	>0.05

Table 4.2 Student's *t* test analysis of differences between varying DLL4 density coated Proactive® beads, anti-6x HIS antibody coated beads and cells for luciferase reporter assay

	Anti-6x HIS antibody bead to cell ratios				
	0.5:1	**1:1**	**2:1**	**5:1**	**Cells**
Low 200 ng DLL4/ million beads					
0.5:1	>0.05	-----	-----	-----	n/a
1:1	-----	***0.080***	-----	-----	***0.070***
2:1	-----	-----	n/a	-----	n/a
5:1	-----	-----	-----	n/a	n/a
Medium 600 ng DLL4/ million beads					
0.5:1	***<0.05***	-----	-----	-----	***<0.05***
1:1	-----	>0.05	-----	-----	>0.05
2:1	-----	-----	>0.05	-----	>0.05
5:1	-----	-----	-----	>0.05	>0.05
High 2000 ng DLL4/ million beads					
0.5:1	***<0.05***	-----	-----	-----	***<0.05***
1:1	-----	***<0.05***	-----	-----	***<0.05***
2:1	-----	-----	***0.063***	-----	***<0.05***
5:1	-----	-----	-----	***<0.05***	***<0.05***

Table 4.3 Fold differences in luciferase activity between high DLL4 density coated Proactive® beads, anti-6x HIS antibody coated beads and cells

	High 2000 ng DLL4/million bead to cell ratios			
	0.5:1	**1:1**	**2:1**	**5:1**
Anti-6x HIS beads	1.69	1.66	1.30	1.64
Cells	1.90	2.06	1.84	2.35

Figure 4.1 Summary of Notch signaling assays utilized in the study. (1) Image acquisition was used to assess qualitative differences in cell morphology using the classic myotube inhibition assay. (2) Differences in fluorescence representing intracellular Notch were assessed through fluorescence microscopy and flow cytometry analysis. (3) Notch target gene expression levels were used to demonstrate Notch signaling in a more sensitive manner. (4) Quantitative differences in CBF1 promoter activity as indicated by the CBF1-luciferase reporter assay confirmed Notch activation with Notch functionalized microbeads.

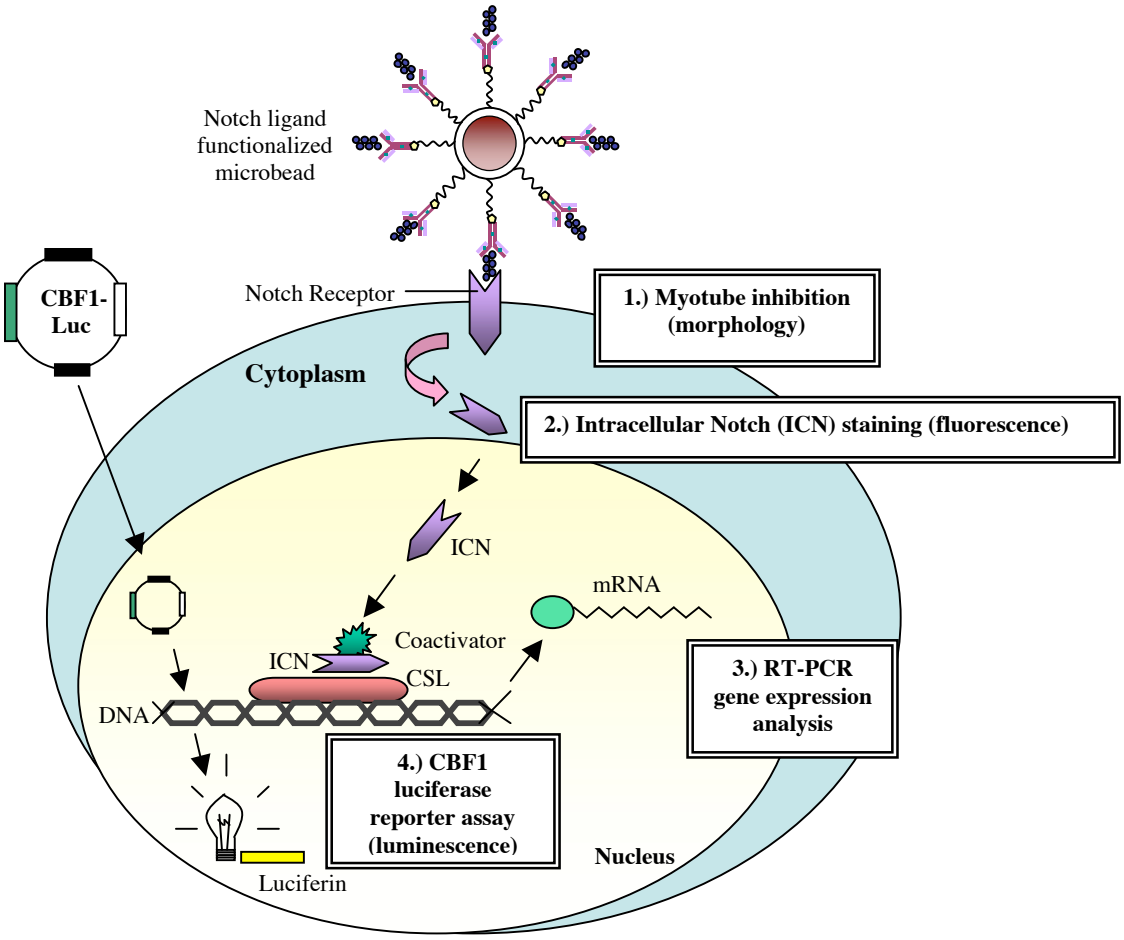


Figure 4.2 Surface immobilization of DLL4 inhibits myotube formation in C2C12 cells. Myotube formation is inhibited in the presence of immobilized DLL4. 10x objective phase contrast images were taken on Day 6 with (a) 0 μg/ml DLL4 (b) 1μg/ml DLL4 (c) 1.5 μg/ml DLL4. Polystyrene 96 well plates were coated with ligand for 3 hours at 37°C and washed subsequently with PBS to remove unattached ligand. C2C12 cells were seeded and incubated for 6 days before image acquisition.

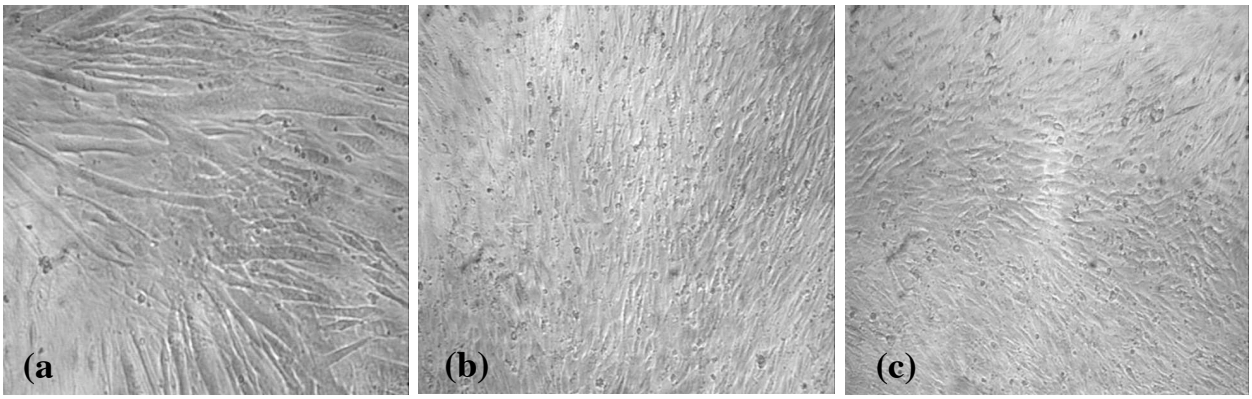


Figure 4.3 Myotube formation is inhibited in the presence of immobilized DLL4. 10x objective phase contrast images were taken on Day 6 with (a) no DLL4 (b) soluble 1 μg/ml DLL4 (c) immobilized 1 μg/ml DLL4. Polystyrene 96 well plates were coated with ligand for 2 hours at 37°C and washed subsequently with PBS to remove unattached ligand. C2C12 cells were seeded and incubated for 6 days before image acquisition.

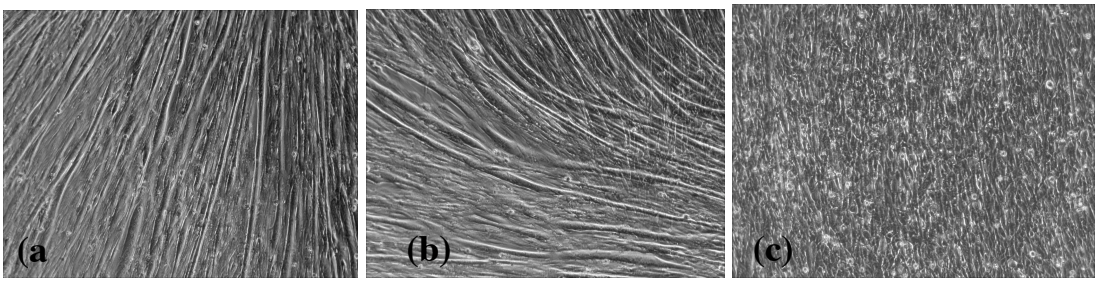


Figure 4.4 Neutravidin and biotinylated anti-6x HIS antibody immobilization do not affect myotube inhibtion. 10x objective phase contrast images were taken on Day 6 with (a) cells only (b) 2 μg/ml immobilized neutravidin (c) 2 μg/ml immobilized biotinylated anti-6x HIS antibody DLL4 (d) 2 μg/ml immobilized DLL4 (e) 2 μg/ml immobilized neutravidin-biotinylated anti6x HIS antibody-histidine tagged DLL4. For neutravidin-biotinylated anti-6x HIS-DLL4 condition, wells were blocked with 1% BSA at 37°C following the antibody incubation. C2C12 cells were seeded and incubated for 6 days before image acquisition. Red arrows indicate myotube formation.

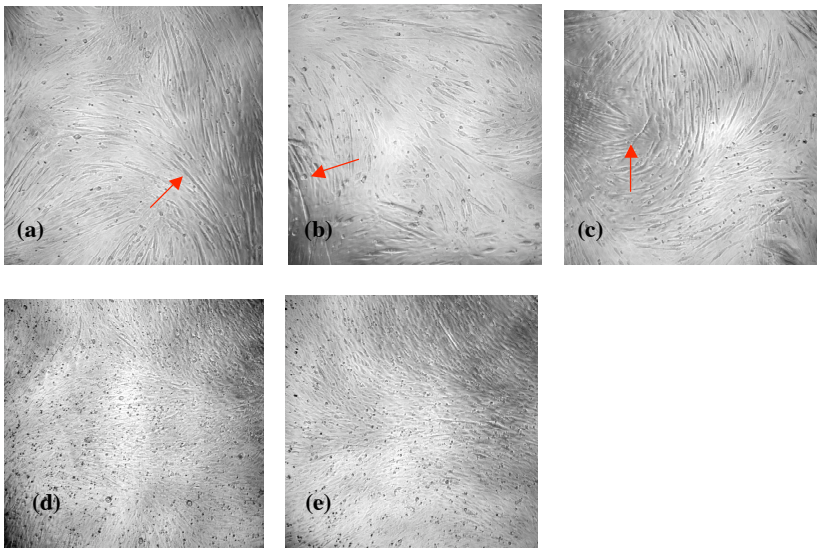


Figure 4.5 DLL4 functionalized microbeads induce Notch signaling in C2C12 cells. Myotube formation is inhibited in the presence of DLL4 coated microbeads. 20x objective phase contrast images were taken on Day 6 with (a) 1:1 uncoated bead to cell ratio (b) 5:1 uncoated bead to cell ratio (c) 1:1 DLL4 coated bead to cell ratio (d) 5:1 DLL4 coated bead to cell ratio

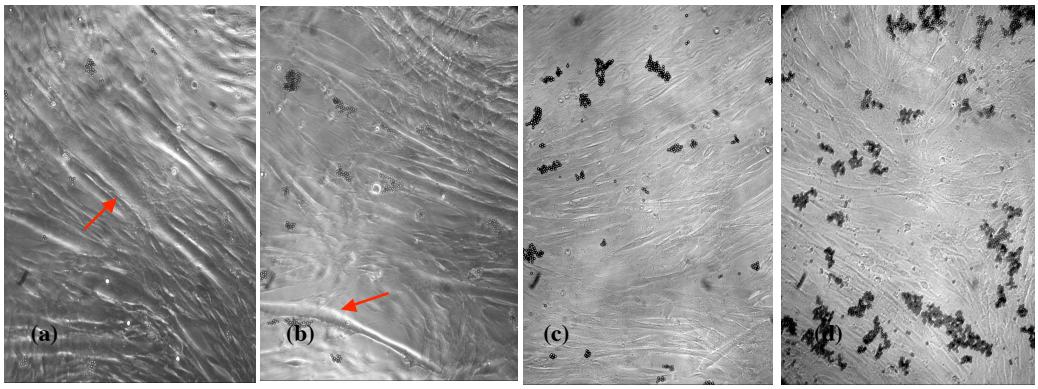


Figure 4.6 Basal levels of Notch signaling in C2C12 myoblasts. C2C12 myoblasts were seeded at 75000 cells/poly-l-lysine coated coverslips one day prior to staining. Cells were then fixed, permeabilized and stained with anti-human/mouse ICN antibody with a FITC conjugated secondary antibody and DAPI to show nucleus. Myoblasts are shown below under (A) phase contrast microscopy and (B) fluorescence microscopy. Myoblasts stained with secondary antibody only are also shown under (C) phase contrast microscopy and (D) fluorescence microscopy. SK-N-MC neuroepithelioma cells are known to always express intracellular Notch. These cells were also prepared as described above and are shown below under (E) phase contrast microscopy and (F) fluorescence microscopy. FITC represents intracellular Notch domain.

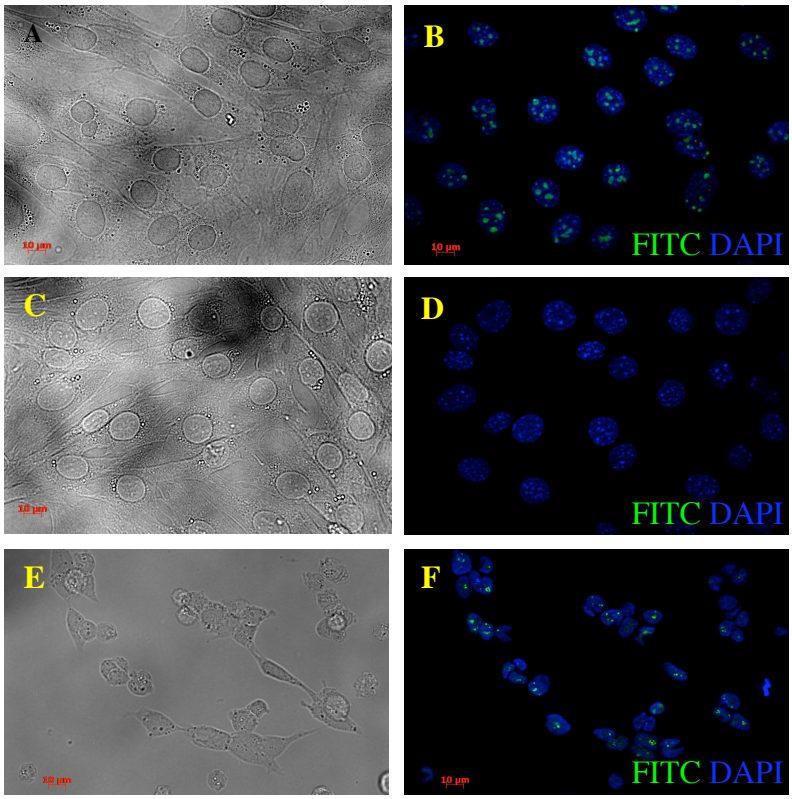


Figure 4.7 Basal levels of Notch signaling in R1 embryonic stem cells. R1 cells were seeded at 20000 cells/poly-l-lysine coated coverslips two days prior to staining. Cells were then fixed, permeabilized and stained with anti human/mouse ICN antibody with a FITC conjugated secondary antibody and DAPI to show nucleus. R1 cells are shown below under (A) phase contrast microscopy and (B) fluorescence microscopy. Myoblasts stained with secondary antibody only are also shown under (C) phase contrast microscopy and (D) fluorescence microscopy. SK-N-MC neuroepithelioma cells are known to always express intracellular Notch. These cells were also prepared as described above and are shown below under (E) phase contrast microscopy and (F) fluorescence microscopy. FITC represents intracellular Notch domain.

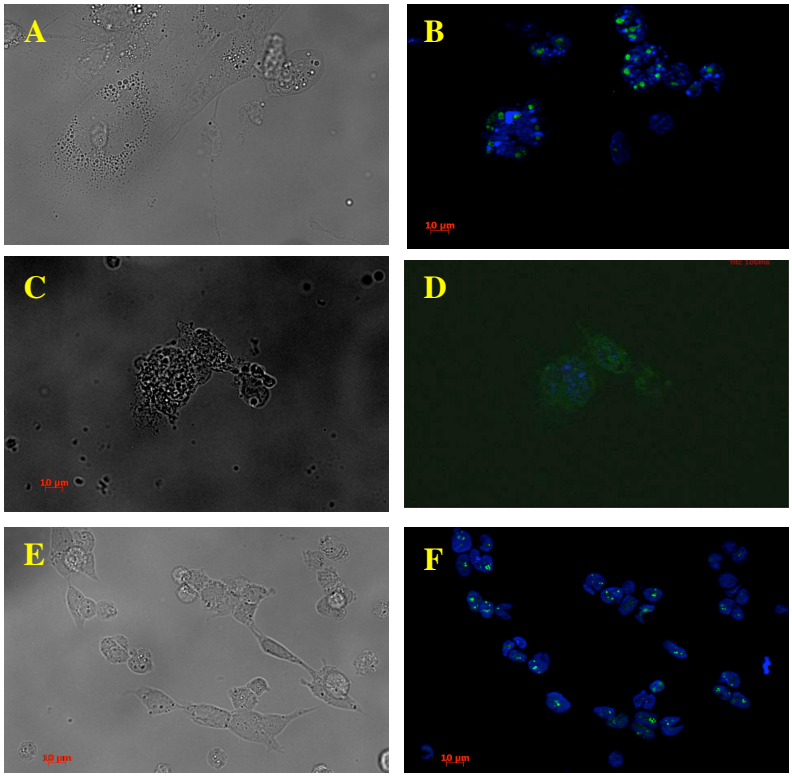


Figure 4.8 Basal levels of Notch target gene expression in R1 ES cells evident after one week of culture. RNA was collected from different timepoints of R1 ESCs, treated for DNA contamination and converted to cDNA. 2 μg of RNA used for cDNA synthesis and RTPCR. Primers for beta actin and HES1 were used to determine levels of Notch signaling over time. Gels were stained with ethidium bromide before imaging. The study was performed with an n=3.

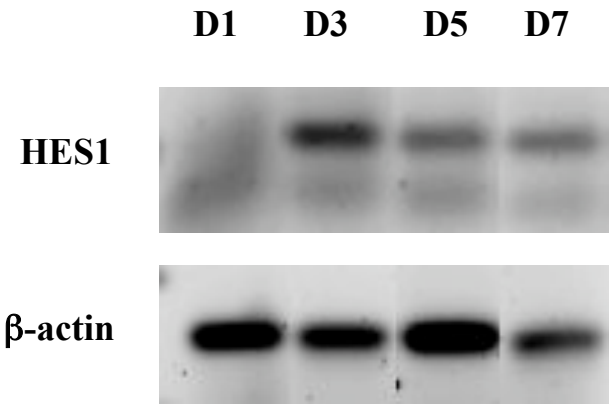


Figure 4.9 Low bead to cell ratios increase Notch target gene expression in C2C12 cells after 2 day incubation. C2C12 cells were incubated with DLL4 functionalized microbeads and uncoated beads for 2 days before RNA isolation and purification. RNA was treated for DNA contamination, converted to cDNA and used for real time RTPCR using gene specific primers, HES1 and beta actin. Values represent an average of at least an n=2 and were normalized to cells only expression. Bars represent standard error.

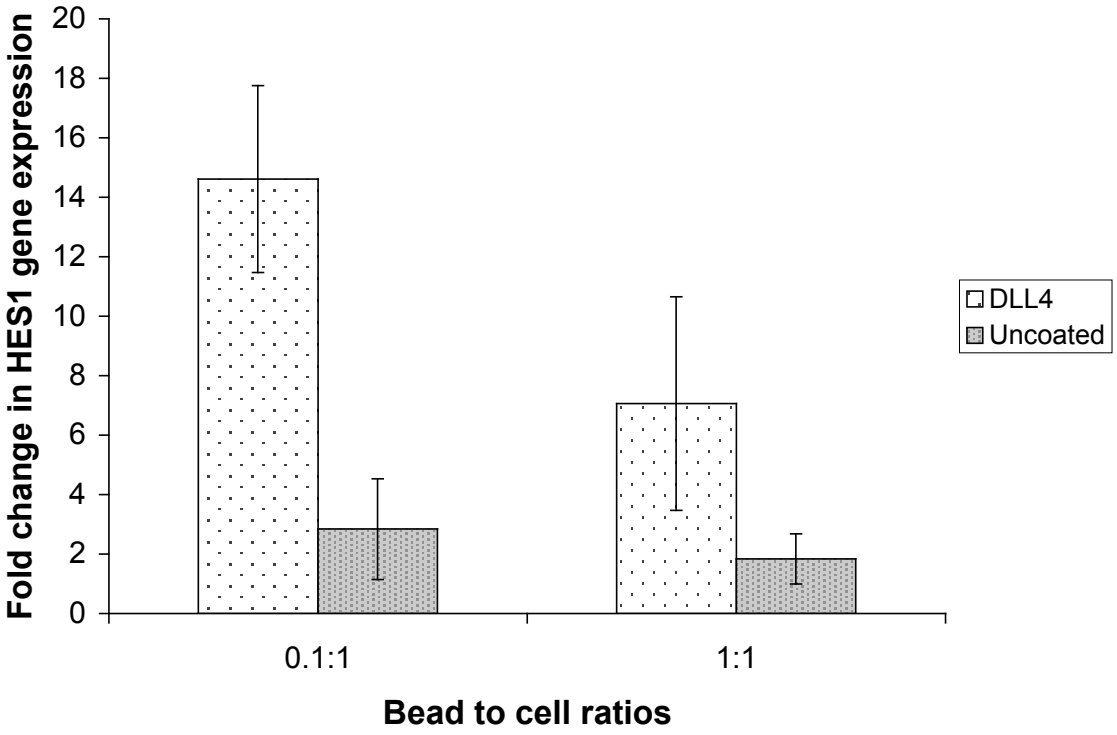


Figure 4.10 Low DLL4 functionalized bead to cell ratios result in HES1 gene expression in Day 6 myoblasts. DLL4 functionalized microbeads were added at varying ratios to C2C12 myoblasts (seeded at 100000 cells/well). RNA was collected after 6 days of incubation, treated for DNA contamination and converted to cDNA. Primers for beta actin and HES1 were used to determine levels of Notch signaling over time using RTPCR analysis. Gels were stained with ethidium bromide before imaging. The study was performed with an n=3.

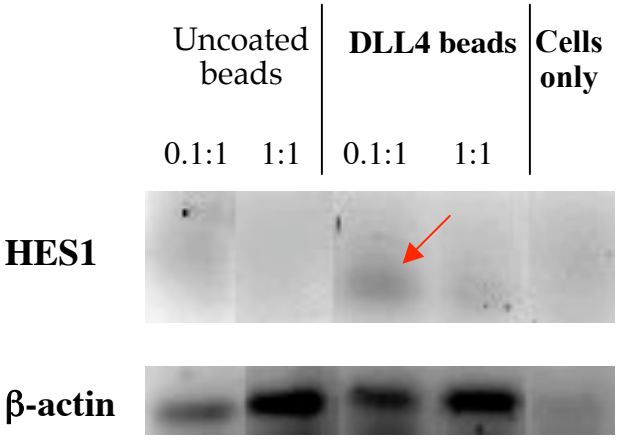


Figure 4.11 Proactive® beads functionalized with DLL4 upregulate Notch gene expression in C2C12 cells after 4 hr of incubation. C2C12 cells were incubated with DLL4 functionalized microbeads and uncoated beads with 0.5 to 1 bead to cell ratio for 4 hr before RNA isolation and purification. RNA was treated for DNA contamination, converted to cDNA and used for real time RTPCR using gene specific primers, HERP2, HES1 and beta actin. Values represent an average of at least an n=3 and were normalized to cells only expression.

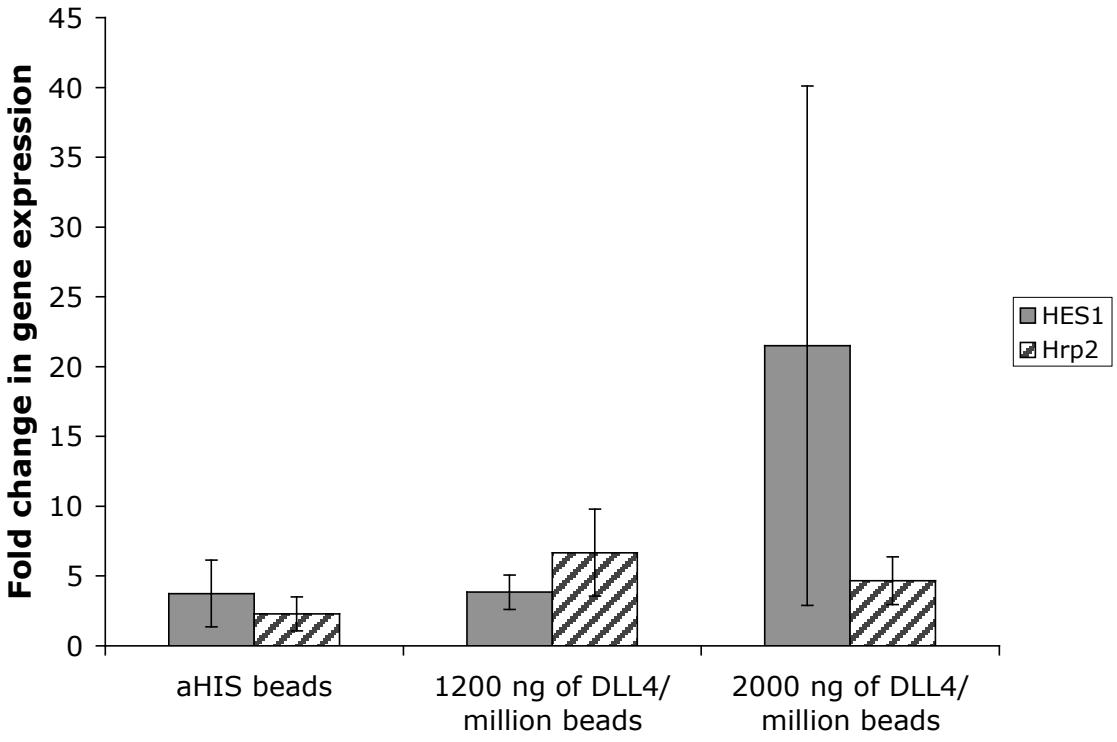


Figure 4.12 DLL4 functionalized Biotin Binder Kit beads result in increased luciferase gene activation in C2C12 myoblasts. C2C12 cells were seeded one day prior to transfection at 100000 cells/well. Cells were then transfected with 4xwtCBF1Luc plasmid and 1 μg/ml using Exgen 500 as per manufacturer's instructions. Beads were functionalized with 100 ng of anti-6x HIS antibody and 1 μg of DLL4 per 10 million beads and added to cells after 24 hr of transfection. Luciferase activity was assessed after 24 hr and corrected to protein concentration using BCA assay. Data represents averages of an n=3 with data bars representing standard error. Cells without beads had an average value of 2318642 +/- 45303.8 RLU / mg of protein.

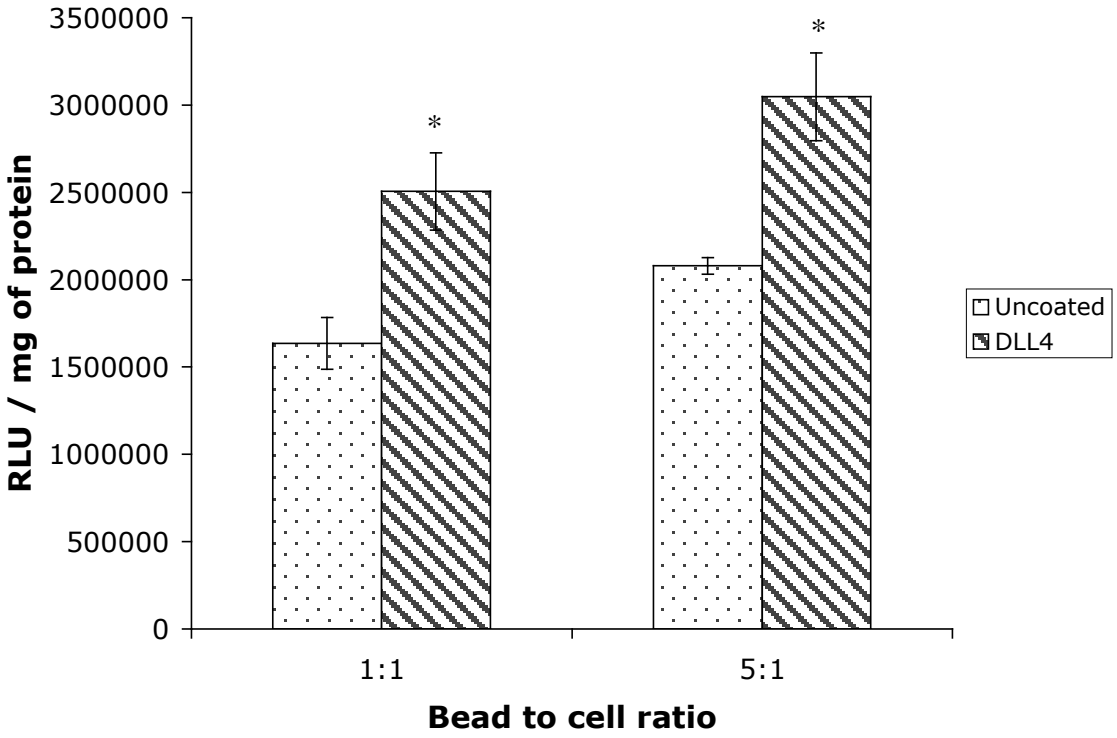
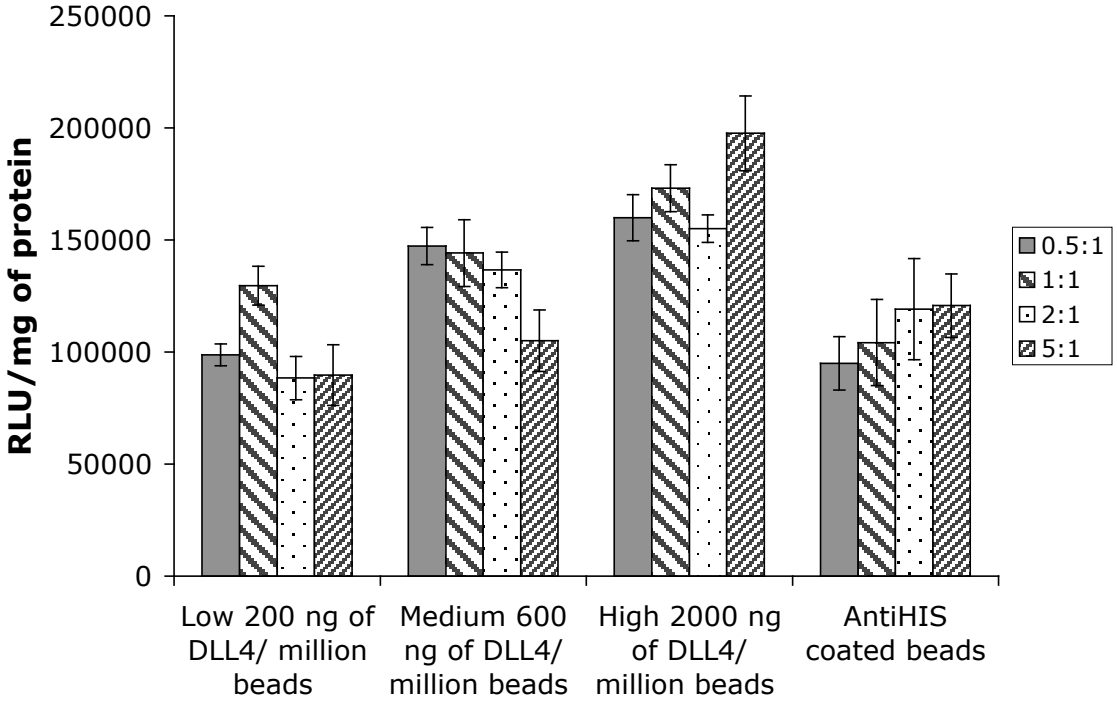


Figure 4.13 DLL4 functionalized Proactive® beads result in increased luciferase gene activation in C2C12 myoblasts. C2C12 cells were seeded one day prior to transfection at 100000 cells/well. Cells were then transfected with 4xwtCBF1Luc plasmid and 1 μg/ml using Exgen 500 as per manufacturer's instructions. Beads were functionalized with predefined amounts of anti-6x HIS antibody and indicated values of DLL4 per 10 million beads at varying ratios and added to cells after 24 hr of transfection. Luciferase activity was assessed after 24 hr and corrected to protein concentration using BCA assay. Data represents averages of an n=3 with data bars representing standard error. Cells without beads had a 84167+/- 21630.2237 RLU/mg of protein value.



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CHAPTER FIVE

T Cell Differentiation Using Notch Ligand Functionalized Beads

5.1 INTRODUCTION

T-cell development is comprised of a series of complex interactions that take place both in the bone marrow and thymus and ultimately results in the formation of CD4+ and CD8+ T cells that are capable of recognizing MHC class II or class I molecules respectively, informing the immune system of extracellular or intracellular infections. Hematopoietic progenitors migrate to the thymus, via the blood and undergo dif-ferentiation into T cells through specific and complex microenvironmental signaling. The expression of ligands and molecules in the thymic microenvironment are directly responsible for the proliferation, adhesion, migration, and selection that these progenitors undergo during T-cell maturation (Anderson et al., 2000; Germain 2002; Goldsby 2003) Several of the signals necessary for thymocyte development and survival, including Notch signaling and MHC-TCR interactions, have been characterized through overexpression, gain-of-function, loss-of-function, and transfection studies (Germain 2002; Maillard et al., 2005).

Notch signaling is known to be well conserved throughout evolutionary development in a variety of organisms. The role of Notch signaling in the regulation of differentiation and self-renewal in systems, such as hematopoiesis and myogenesis, has also been well characterized (Germain 2002; Han et al., 2000; Karanu et al., 2000; Maillard et al., 2005; Parreira et al. 2003; Radtke et al. 2004; Karanu et al., 2000; Tan-Pertel et al., 2000; Varnum-Finney et al., 2003). Specifically in T-cell lymphopoiesis, Notch signaling has been shown to be a necessary criterion. In the absence of Notch

signaling, lymphopoiesis only occurs along the B cell lineage while the presence of Notch ligands on the surface of stromal cells provide signals necessary for T-cell generation (Parreira et al. 2003, Radtke et al. 2004). Notch receptors and the Delta and Jagged families of Notch ligands are tightly regulated in their expression both in the bone marrow and thymus to achieve a unique balance of lymphocyte development (Maillard et al. 2005; Parreira et al. 2003; Radtke et al. 2004). The expression of all four receptors by the developing thymocytes and the supportive thymic stroma has been previously characterized while expression of Notch ligands, Delta-like ligands 1 and 4 (DLL1 and DLL4), has been shown to occur in the thymic stroma (Parreira et al. 2003). However, studies assessing the importance of cell–ligand ratio as well as duration of ligand presentation have not been explored in detail.

Our ultimate goal is to develop functionalized biomaterials and synthetic microenvironments to efficiently generate T cells from hematopoietic stem cells (HSCs). Eventually, this could lead to the production of CD4+ and CD8+ cells that could be used for therapeutic purposes. Current systems to generate T cells *in vitro* have largely relied on coculture of stem cells with either fetal thymus isolated from mouse (fetal thymic organ culture, FTOC) or more recently with bone marrow derived stromal cells (OP9 cells) retro-virally transfected with the Delta and Jagged families of Notch ligands (Dallas et al. 2005; Hozumi et al. 2003; Hozumi et al. 2004; La Motte-Mohs et al. 2005; Lehar et al. 2005; Schmitt et al. 2002; Schmitt et al. 2004a). These systems, although effective, are complex, require genetic manipulation of stromal cells and most importantly do not allow quantitative analysis of the effects of ligand to stem cell ratio or the duration of Notch signaling on T-cell differentiation. Tissue engineering of T cells from progenitor populations in a high-throughput and efficient manner could benefit from synthetic approaches involving biomaterial-directed Notch ligand presentation.

Here, we report a synthetic Notch signaling system using ligand-functionalized magnetic microbeads (artificial stromal cells) that can be used to evaluate how Notch ligands, specifically DLL4, presented through a biomaterial surface affect T-cell differentiation and to eventually develop a high throughput strategy to engineer T cells from hematopoietic progenitor populations. We functionalized microbeads with DLL4 using biotin-streptavidin chemistry and antigen–antibody coupling and demonstrated their functionality through T-cell commitment studies. Flow cytometry analysis showed that Notch ligand functionalized beads are sufficient to commit bone marrow hematopoietic stem cells (BMHSCs) to the T-cell lineage using the OP9 coculture system. We additionally found that stem cell–stromal cell contact is not a necessary event for T-cell commitment with DLL4 based Notch signaling. Such a bead-based artificial signaling system could allow us to quantitatively study the effects of ligand density and signaling duration thereby providing further insights into the individual roles of the Delta and Jagged families of Notch ligands in T-cell differentiation and ultimately aid in the development of efficient technologies for the production of T cells for therapeutic applications.

5.2 MATERIALS AND METHODS

5.2.1 Conjugation of Notch ligand DLL4 to microbeads

The Notch ligand delta-like ligand 4 (DLL4) was conjugated to magnetic microbeads using previously published methods for microbead functionalization (Maus et al., 2003; Trickett et al., 2002; Trickett et al., 2003). Briefly, biotinylated antibodies specific for a histidine tag on recombinant DLL4 were bound to streptavidin-coated superparamagnetic polystyrene microbeads. Biotin Binder Kit microbeads (Dynalbiotech,

Brown Deer, WI) were washed and incubated with anti-6x HIS tag antibody (R&D Systems, Minneapolis, MN) at 1 μg/mL for 30 min at room temperature. After incubation, beads were again washed and further incubated with the HIS-tagged DLL4 protein (R&D Systems, Minneapolis, MN) at 2–4 μg/mL for 30 min at room temperature. Beads were washed and stored at 4-8ºC for future use.

5.2.2 Bone marrow hematopoietic stem cell isolation and culture for *in vitro* T-cell development

Stem cells were cultured with DLL4 functionalized microbeads, unmodified OP9 cells and exogenously added growth factors similar to methods described by Hozumi et al (Hozumi et al., 2003). OP9 cells (kind gift from Tammy Reid, Toronto, Canada) were maintained in 20% FBS (Hyclone, Logan, UT), 2.2 g/L sodium bicarbonate (Invitrogen, Carlsbad, CA) and αMEM (Invitrogen, Carlsbad, CA). They were seeded at the appropriate cell density in 24-well plates 1 day before stem cell seeding to achieve 60% confluency. BMHSCs were isolated from 5-week-old female C57BL/6 mice (Jackson Laboratory, Bar Harbor, Maine), using standard femur removal protocols. Lin-ckit+sca-1+ HSCs were isolated using magnetic separation (Miltenyi Biotec, Auburn, CA and Dynal-biotech Brown Deer, WI) and seeded at a density of 2000 cells per well either directly on top of the OP9 cell layer (mixed coculture) or in Transwell™ permeable inserts (Corning, Acton, MA) (insert coculture) to assess if stem cell–stromal cell physical contact is absolutely necessary for T-cell generation or whether paracrine signaling is sufficient. Stem-cell factor (SCF) (Peprotech, Rocky Hill, NJ) and interleukin-7 (IL-7) (Peprotech, Rocky Hill, NJ) were added to the culture at 50 and 10 ng/mL, respectively. DLL4 functionalized or nonfunctionalized Biotin Binder Kit beads (Dynalbiotech, Brown Deer, WI) were added at defined concentrations to sample and

control wells, respectively. For the mixed coculture condition, cells were disrupted on Day 4 and single cell suspensions were filtered through a 40μm filter to remove OP9 cells, which generally form aggregates. Cell suspensions were again seeded on fresh monolayers of OP9 cells for continued culture. Beads, growth factors, and medium were replenished. For insert culture, Transwell™ inserts were removed on Day 4 and placed in wells containing fresh OP9 monolayers and replenished with beads, growth factors, and medium. On Day 8, cells were trypsinized and separated from beads using DNAse (the streptavidin on these beads are conjugated using a DNA linker) as per manufacturer's protocol (Dynalbiotech, Brown Deer, WI).

5.2.3 Flow cytometry

Flow cytometry was performed similar to methods described in Liu et al (Liu and Roy 2005). Cells were washed in FACS buffer (1% bovine serum albumin (Sigma-Aldrich, St. Louis, MO) and 0.05% sodium azide (Sigma-Aldrich, St. Louis, MO)) in PBS twice before staining and blocked for nonspecific binding using anti-mouse CD16/CD32 Fc Block (BD Pharmingen, San Diego, CA) for 10 min at 4-8°C. Staining with antibodies against stage specific T-cell markers was performed at concentrations of 1 μg/100mL of FACS buffer and washed before staining with secondary fluorescently labeled antibodies, if necessary. Isotype controls (eBioscience, San Diego, CA) were used as negative controls. All cells were washed in FACS buffer twice and suspended in fresh buffer for analysis. FACSCalibur (Becton Dickinson, San Diego, CA) and CellQuest 3.1 software (BD Biosciences, San Jose, CA) were used for data acquisition and analysis. The following antibodies and fluorochromes were used to assess stage-specific T-cell development from BMHSCs: biotinylated CD19 (Sigma-Aldrich, St. Louis, MO), Thy1.2-FITC, and streptavidin-PE. CD19 is a B-cell specific surface marker

while Thy1.2 has been extensively used as an early T-cell marker in differentiation studies (Hozumi et al., 2003; Hozumi et al., 2004; Schmitt et al., 2002; Schmitt et al., 2004a). All antibodies were obtained from BD Pharmingen (San Diego, CA) or eBioscience (San Diego, CA) unless otherwise noted.

5.3 RESULTS

5.3.1 DLL4 functionalized microbeads direct BMHSCs to T-cell lineage in OP9 co-culture systems

To demonstrate the functionality of the DLL4 functionalized microbeads, we added these beads to the well-established OP9 coculture system and examined the effect on T-cell differentiation. The OP9 coculture system has been widely used for lymphoid differentiation applications due to the secretion of lymphoid specific growth factors from OP9 stromal cells (Ciofani et al., 2004; De Smedt et al., 2004; Hozumi et al., 2003; Hozumi et al., 2004; Lehar et al., 2005; Nakano et al., 1994; Nakano 1995; Schmitt et al., 2002; Schmitt et al., 2004a; Schmitt et al., 2004b). However coculture of stem cells with OP9 cells without the presence of any notch signaling exclusively generates B cells. Recently, several groups have transfected both the original stem cell source and supportive OP9 cells with various Notch ligands and observed the emergence of cells of the T-cell lineage (De Smedt et al., 2004; Hozumi et al., 2003; Hozumi et al., 2004; Lehar et al., 2005; Schmitt et al., 2002; Schmitt et al., 2004a; Schmitt et al., 2004b). We added the DLL4 functionalized microbeads to the OP9 coculture system and analyzed the cultures for the expression of B and T-cell specific surface markers, CD19 and Thy1.2, respectively. We also examined the effect of cell–cell contact on T-cell differentiation using the microbead and OP9 system. Transwell™ permeable inserts were used to prevent cell–cell contact with the supportive OP9 layer on the culture well and BMHSCs

on insert. Insert culture was compared to conditions where the BMHSCs were seeded directly on the OP9 monolayer. Bead-to-cell ratio of 1:1 was used for both conditions and streptavidin-coated beads were used as negative controls. Cultures with DLL4 functionalized beads gave rise to both Thy1.2+ cells and CD19+ cells while streptavidin-coated bead culture resulted in CD19+ cells only. This held true for both insert and coculture conditions. In comparison to coculture conditions, there was a larger percentage of Thy1.2+ cells in insert culture conditions, 59.7% vs. 33.4% (**Figure 5.1**). In both insert and coculture systems, functionalized bead conditions resulted in the commitment of both T and B cells. The sizable B-cell population is most likely due to the nonuniform distribution of the functionalized beads and the inability of some BMHSCs to "see" the Notch signal. B-cell differentiation efficiencies using streptavidin-coated control beads (no DLL4 on surface) was comparable for both insert and coculture conditions, 79.9% vs. 73.8%, respectively. During the 1 week differentiation process, the original stem-cell population underwent a proliferation of at least 2.0-fold for all conditions (data not shown).

5.3.2 Defined ratios of DLL4 functionalized microbeads can be used for T-cell differentiation

To further investigate the quantitative effects of the DLL4 Notch ligand on T-cell commitment, we added the DLL4 functionalized microbeads at various concentrations (1:1 and 5:1 bead-to-cell ratio) and observed the lymphocyte specific marker expression after 1 week of culture, similar to the procedure outlined earlier. DLL4 functionalized beads, at a 1:1 bead-to-cell ratio, resulted in a significant amount of Thy1.2 expression while a bead-to-cell ratio of 5:1 gave rise to little if any Thy1.2 expression (**Figure 5.2**). Low T-cell differentiation efficiencies observed in the 5:1 functionalized bead-to-cell

ratio condition may be a result of the high bead concentration in culture. It is likely that the beads have an inhibitory effect on proliferation of progenitors at high densities.

5.4 DISCUSSION

Recent studies have indicated a significant role for the Notch ligand DLL4 in T-cell commitment and development. Hozumi et al. demonstrated the presence of Thy1.2+ cells from fetal liver hematopoietic progenitors using OP9 cells stably transfected to express DLL4 (Hozumi et al., 2004). These differentiated cells were also shown to undergo VDJ recombination and express CD25 and/or CD44, indicating the maturation to the double negative 3 (DN3) stage of T-cell development. Freitas et al. conducted a recent study where the overexpression of DLL1 or DLL4 in hematopoietic cells was shown to result in the induction of mature single positive (SP) T cells in athymic mice (de La Coste et al., 2005; de La Coste et al., 2006; Hozumi et al., 2004). These studies, however, involve mixed coculture with genetically modified cells and hence suffer from two fundamental drawbacks: (a) it is difficult to quantitatively study the effects of ligand–cell ratio and ligand interaction duration and (b) ultimately high throughput scale up of such mixed coculture process for large scale production of therapeutic cells from stem cells would be challenging.

The efficient generation of T cells has been achieved through several stem cell-stroma based coculture systems (La Motte-Mohs et al., 2005; Lehar et al., 2005; Poznansky et al., 2000; Schmitt et al., 2004). However, limited studies have been reported in generating T cells using biomaterial-based concepts. Poznansky et al. utilized tantalum-coated matrices seeded with mouse thymic stroma to provide the necessary molecular cues to commit human hematopoietic stem cells to the T-cell lineage (Poznansky et al., 2000). Despite the high efficiency of CD3+ T-cell generation, the

inherent design of this system hinders the isolation of a pure population of T cells, making the large scale generation of T cells difficult.

The OP9-DLL1 Notch signaling, first reported by Zuniga-Pflucker and colleagues also utilizes a mixed coculture based design to provide direct cell–cell signaling necessary to commit stem cells to the T-cell lineage (Zuniga-Pflucker 2004). One fundamental limitation of this system, however, is the dependence of OP9 based systems on transfected cells for Notch signaling. The transfection of stroma cells for different Notch ligands can become cumbersome and interfere with normal genetic expression of the OP9 cell (Lehar et al., 2005). In addition, the inherent design of the current coculture system makes large-scale T-cell generation difficult, specifically for 3D studies in biomimetic environments. Our design addresses the abovementioned limitations by (a) providing Notch signals through immobilization of DLL4 on microbeads (artificial stromal cells) thereby creating a highly controllable, predesigned thymic microenvironment and (b) by physically separating the OP9 cells from the stem-cell population during differentiation, which enable us to obtain a purer population of T cells. The magnetic nature of the bead also facilitates the retrieval of T cells eliminating the difficulties involved with obtaining a pure population.

In this study, we have shown that microbeads functionalized with the Notch ligand DLL4 can be used as artificial stromal cells to trigger Notch signaling in myoblasts and commit BMHSCs to the T-cell lineage in both coculture and insert culture systems in a quantitative manner. This study is one of the first to investigate the role of DLL4 in T-cell differentiation using a synthetic, biomaterial-based signaling system. The results demonstrate the promise of a bead-based system in studying the roles of Notch ligands in lymphocyte development and efficiently generating T cells from progenitor cell population using functionalized biomaterials.

To demonstrate the functionality of the microbeads in lymphocyte development, BMHSCs were cultured in the presence of functionalized beads and either physically separated OP9 stromal cells (insert cultures) or mixed coculture conditions. Our results indicate that functionalized beads induced a significant percentage of Thy1.2+ cells in both cultures. However, insert cultures appeared to generate a higher percentage of cells. The reduced surface area in the Transwell™ inserts leads to higher stem cell–stem cell interactions (for the same number of cells plated), which could be responsible for the increased differentiation efficiency. CD19+ B cell differentiation efficiency was similar among both insert and coculture conditions with or without functionalized beads. The presence of some B cells in the Notch functionalized bead culture again indicates nonuniform localization of the DLL4 beads in the wells. Still, the differentiation efficiency of Thy1.2+ cells for at least the insert condition is comparable to the results described by Hozumi et al., where the Notch ligand was provided by OP9 cells transfected for DLL4 (Hozumi et al., 2004).

Finally, to investigate the quantitative effects of DLL4 on lymphocyte development, bead-to-cell ratios of both 1:1 and 5:1 were added in the culture conditions. We found that functionalized bead-to-cell ratios of 1:1 resulted in a significantly higher Thy1.2+ cell differentiation efficiency than the 5:1 bead-to-cell ratio. The reduced differentiation efficiency in the 5:1 functionalized bead-to-cell ratio may be attributed to an inhibitory effect on progenitor proliferation, similar to what has been observed in T-cell activation studies with anti-CD3/anti-CD28 microbeads (Ito et al., 2003). Future studies are needed to elucidate the dose effects of the functionalized microbeads on progenitor proliferation. The aggregation or uneven distribution of the functionalized beads will also need to be prevented through studies where beads and cells are preincubated for an optimal period of time, enabling beads to be prebound to cells before

they are placed in culture. Finally, a more extensive immunophenotype analysis examining the expression of T-cell markers such as CD25, CD44, CD3, CD4, CD8, and a genetic analysis assessing the expression of T-cell specific genes such as CD3ε and pre-Tα are necessary to confirm the emergence of T cells and demonstrate the level of development.

In conclusion, we have established a synthetic biomaterial-based system that can effectively trigger Notch signaling during lymphocyte development from bone marrow-derived stem cells. The system offers the possibility of being quantitative and tunable, enabling the optimization of T-cell generation. This system could further progress our understanding of ex vivo T-cell generation and might allow us to eventually generate functional T cells from stem cells using biomaterial-based approaches.

Figure 5.1 Notch ligand-coated microbeads direct efficient commitment of ES cells to T cells in both insert and mixed coculture systems. Lin-cKit+sca-1+ HSCs were cultured with DLL4 coated or uncoated beads in insert cultures and mixed coculture conditions in the presence of IL-7 and SCF. Cells were harvested and analyzed for CD19 and Thy1.2 expression using flow cytometry. Numbers in quadrants correspond to relative percentages of cells. A) Comparison of Day 8 T-cell differentiation in insert culture from lin-cKit+sca-1+ BMHSC with 1:1 bead-to-cell ratio using uncoated and DLL4 coated beads. B) Representative flow cytometry dot plots of Day 8 T-cell differentiation profiles in insert culture from lin-cKit+sca-1+ BMHSC with 1:1 bead-to-cell ratio. C) Comparison of Day 8 T-cell differentiation in mixed coculture from lin-cKit+sca-1+ BMHSC with 1:1 bead-to-cell ratio using uncoated and DLL4 coated beads. D) Representative flow cytometry dot plots of Day 8 T-cell differentiation profiles in mixed coculture from lin-cKit+sca1+BMHSC with 1:1 bead-to-cell ratio from flow cytometry analysis. All experiments were repeated twice. *indicates $p < 0.05$ compared to DLL4 coated beads using a Student's *t* test.

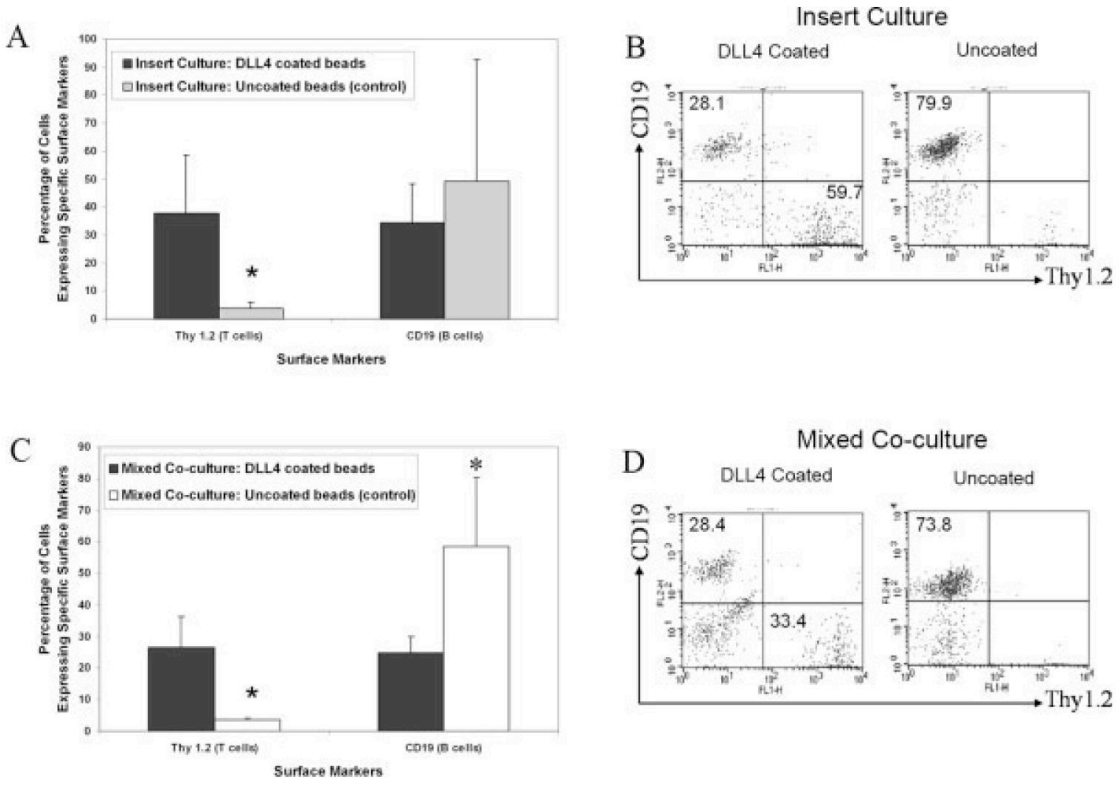
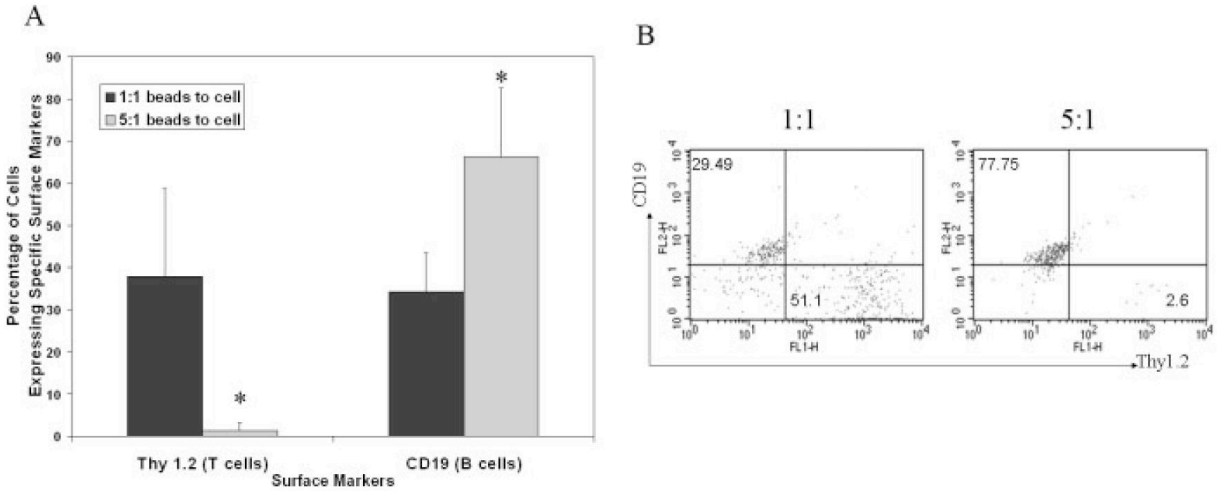


Figure 5.2 Defined ratios of notch ligand-microbead can be used for T-cell commitment. Lin-cKit+sca-1+ HSCs were cultured with DLL4 coated or uncoated beads in insert cultures and mixed coculture conditions in the presence of IL-7 and SCF. Cells were harvested and analyzed for CD19 and Thy1.2 expression using flow cytometry. A) Comparison of Day 8 T-cell differentiation in insert culture from lin- cKit+sca-1+ BMHSC with 1:1 and 5:1 bead-to-cell ratios. B) Representative flow cytometry dot plots of Day 8 T-cell differentiation profiles in insert culture from lin- cKit+sca-1+ BMHSC with 1:1 and 5:1 bead-to-cell ratio. Numbers in quadrants correspond to relative percentages of cells. All experiments were repeated twice. * indicates $p < 0.05$ compared to DLL4 coated beads using a Student's *t* test.



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CHAPTER 6

Conclusions and Future Directions

6.1 SUMMARY

In this study, we have designed and demonstrated a novel approach to ex vivo T cell differentiation, the use of a Notch ligand functionalized microbead. The process of ligand functionalization onto the bead surface has been extensively characterized in terms of bead concentration, incubation time and ligand concentration. Additional studies have demonstrated stability in buffers and serum-supplemented medium for beads without DNA linkers. Low cytotoxicity and ability to quantify ligand density on bead surface are additional benefits of such a bead system. We further demonstrated a dose-dependent response in Notch signaling using qualitative and quantitative assays. Finally, the Notch ligand functionalized microbeads were successfully shown to differentiate hematopoietic progenitors into committed T cell precursors. These results demonstrate the immense promise of such a biocompatible and quantifiable system that can be readily scaled up and tailored to optimize Notch signaling and T cell differentiation efficiency. Efforts in optimizing scaffold parameters for hematopoietic differentiation also illustrate the ability to tailor a microenvironment for high throughput production of hematopoietic progenitors.

6.1.1 Conclusions and future directions on bead fabrication, characterization and optimization studies

Chapter 3 results illustrate successful Notch ligand functionalization on magnetic microbeads using an in depth optimization process. Our goal in microbead design was to

utilize a highly directional and robust binding scheme that could be both quantified and tailored (in terms of surface ligand density) for Notch signaling applications and scaled up for T cell differentiation studies. For these reasons, we chose commercially available 4.5 μm streptavidin coated super paramagnetic microbeads that were designed for bead-cell separation. Use of biotinylated anti-6x HIS antibody and histidine tagged DLL4 exploited the well-characterized and high strength streptavidin-biotin and antibody-antigen interactions. The original microbead design also included bead-cell separation capability, namely the presence of a DNA linker that could be subsequently cleaved and thus result in the removal of beads from cells with the addition of DNase. Bead characterization using immunofluorescence and flow cytometry analysis indicated a saturation of bead surface well below theoretical values with optimum protein binding at high bead concentration and low incubation times for both anti-6x HIS antibody and histidine tagged DLL4. Surface ligand density was also quantified and shown to be modifiable for a wide range of protein concentrations. Studies indicate rapid immobilization of proteins for similar bead systems (Curtsinger et al., 1997). Further characterization studies involving stability in varying buffers at different temperatures, however, revealed a severe constraint of the original design. Little to no DLL4 could be observed on the surface of the beads after only one hour at 37°C in serum-supplemented or serum-free medium, despite the high binding strength of the streptavidin-biotin and antibody-antigen bonds. We hypothesized that the presence of the DNA linker enabled rapid ligand removal at 37°C with the omnipresent nature of DNase. Thus, our bead design was refined to a simple streptavidin coated magnetic bead. These beads gave far superior stability in all buffers tested at both 4 and 37°C with no effect of the presence of serum. One concern, however, remains the nearly 50% reduction of the surface ligand density in PBS at 4°C after just one day of incubation. Future studies that characterize

the stability of both the DLL4 ligand and the biotinylated antibody will enable a better understanding of the total strength of the combined bonds. Although the functionalized beads are stored at conditions suggested by the manufacturer, it is also possible that the PBS buffer is not ideal for storage of protein functionalized microbeads. Additional studies where various concentrations of BSA protein in PBS can be used for microbead storage and tested for any improvement in bead stability.

Cytotoxicity results from week long bead-cell studies at various bead to cell ratios indicated low cytotoxicity with some proliferative effects. Cytotoxicity has been observed for bead-cell studies in cells such as T cells and fibroblasts, however, seems to be unique to cell type tested. Also, length of incubation time has an effect on cytotoxicity. Longer incubation times for the cells tested may result in similar observations. Future studies should include high bead to cell ratios in different cell types with longer incubation periods. Incubation period is a particularly sensitive aspect for the bead system due to the longer incubation times necessary for human T cell differentiation applications.

Perhaps the most interesting and least characterized aspect of the system is the intrinsic nature of magnetic beads and how it affects protein presentation. During cell-bead studies, bead aggregation at high densities and thus, lower effective ligand presentation was observed for both uncoated and Notch functionalized beads alike (presence of ligand did not affect aggregation degree). Such aggregation might be prevented with the addition of detergents such as Tween20 that prevent protein-protein interactions. Vortexing may decrease aggregation, as well. Future studies exploring the effects of Tween20 and vortexing on bead aggregation (and at the same time preserving protein structure and function and not affecting cell viability) should be performed. Aggregation may also be a result of the magnetic nature of the bead. It may be likely that

beads without magnetic particles aggregate to a lower degree. Future studies with nonmagnetic beads should be conducted to study the effect if any of magnetic particles in bead aggregation.

Finally, how successful the presentation method, two proteins immobilized on beads, is in being recognized and resulting in a signal deserves future studies and will be a key player in determining the effectiveness of the system. Several studies have alluded to the importance of chain mobility in protein binding, most likely since this more closely resembles the surface of a cell (Curtsinger et al., 1997; Maus et al., 2003). Future studies where a more direct scheme (directly conjugated DLL4 on bead surface) is used would aid in the understanding of the beneficial effects, if any, of having a more mobile binding scheme. Also, chain length may play a role in cell-bead binding. Further studies where only DLL4 is functionalized on the bead surface can better explain the importance of chain length.

6.1.2 Conclusions and future directions on qualitative and quantitative characterization of dose-dependent Notch signaling

In Chapter 4, we performed a series of assays directed at characterizing the dose-dependent effect, if any, of Notch functionalized microbeads. We hypothesized that the degree of Notch signaling could be adjusted by varying the amount of ligand density on bead surfaces and/or amount of beads added to cells. Several studies have reported a dose-dependent effect of Notch signaling in terms of Notch target gene expression, luciferase reporter activity, and immunophenotype analysis (Dallas et al., 2005; Delaney et al., 2005; Ohishi et al., 2002; Schmitt et al., 2004). More importantly, the degree of Notch signaling has been shown to be instrumental in deciding the fate of developing lymphocyte progenitors. Studies have shown a Notch signaling threshold where lower

amounts of Notch signaling enable B cell differentiation, medium amounts allow NK cells, and high concentrations permit T cell differentiation (Dallas et al., 2005; Delaney et al., 2005; Lehar and Bevan 2005; Ohishi et al., 2002; Schmitt et al., 2004). The dose-dependent quality of Notch signaling is thus, directly pertinent in differentiation efficiency. The myoblast system was used primarily due to ease and extensive characterization in literature. Qualitative studies where morphology was assessed (Notch signaling inhibits myotube formation) confirmed Notch signaling due to bead. Next, more quantitative approaches, intracellular Notch signaling, RTPCR analysis and luciferase reporter assay were all used to quantitatively characterize Notch signaling efficiency at a more molecular level. (Both beads were used for all assays except the qualitative assay where only DNA linker beads were used. Beads showed similar results in all assays.) Intracellular Notch signaling studies showed basal levels of activated Notch in both myoblasts and embryonic stem cells (which is confirmed in literature) and did not seem to be sensitive enough to detect differences, if any, in the addition of Notch functionalized beads (Iso et al., 2001, Nemir et al., 2006). Gene expression analysis, however, indicated some promising results for both bead types at early and late timepoints in myoblasts. The two day timepoint exhibited a 5 fold increase in HES1 gene expression for the DNA linker beads and a 3 fold increase in HERP2 gene expression for Proactive® beads at bead to cell ratios of 0.1:1 and 0.5:1, respectively. These results are on the same scale as similar studies monitoring Notch target gene expression but with immobilized Notch ligands (Dallas et al., 2005; Delaney et al., 2005; Varnum-Finney et al., 2000). In these studies, high bead to cell ratios failed to increase Notch target gene expression for both bead types. CBF1 luciferase reporter assay showed similar results for both bead types. Only the Proactive® bead, however, produced statistically significant differences at very high ligand densities between both Notch

functionalized beads and anti-6x HIS antibody coated beads and Notch functionalized beads and cells.

Several issues need to be further studied to better understand the RTPCR and luciferase reporter results. These results do indeed illustrate dose responsive behavior for myoblasts. Whether the ligand density or a quality of the bead system is activating the Notch receptor needs to be further studied. We have previously demonstrated the low stability of the DNA linker beads from flow cytometry analysis. If the DLL4 has been cleaved from the surface of the beads but Notch activation has resulted, two scenarios may have occurred. In the first scenario, the cleaved DLL4 could have associated with more DLL4, formed an aggregate and resulted in the clustering of Notch receptors and Notch signaling. Another possible option is that for the limited amount of time the ligand remains functionalized, cells receive a signal which is sufficient to produce the results shown using the quantitative assays. Further studies testing the medium for the presence of DLL4 and DLL4 aggregates and studies assessing the effect of incubation time on gene expression and luciferase assays are necessary to better understand how the DNA linker beads produced Notch signaling. In addition, it is possible that the DLL4 is being released from the Proactive® beads. Another observation from the studies was the effect of bead to cell ratio. Results were generally poor in terms of gene expression and luciferase expression for high bead to cell ratios. Luciferase results, in fact, indicated little to no statistical difference between bead to cell ratios for all densities tested. These results can be explained by the aggregation effect described in the previous section. When aggregation takes place, the effective ligand density delivered is far less. Future studies preventing aggregation of beads and possibly distributing beads evenly on well surfaces need to be performed to increase effective Notch ligand density that is in fact being seen by cells. Finally, if observed closely, intracellular Notch staining, gene

expression analysis and luciferase reporter assay all indicate high basal levels of Notch activity for myoblasts. Other studies have similarly shown inherent Notch signaling in myoblasts (Iso et al., 2005). The myoblast system was chosen based on its ease and well-established nature. The system, however, does not seem ideal for our purposes. An available or transfected cell line where Notch receptors are present and Notch ligands are not present, must be utilized to accurately assess the effect of the functionalized Notch ligand beads. This will eliminate any basal levels of Notch signaling due to cell-cell contact. Also, unless the functionalized beads can be evenly distributed to cover the entire well surface, bead-cell contact cannot be guaranteed.

6.1.3 Conclusions and future directions on T cell differentiation using Notch functionalized microbeads

In Chapter 5, we demonstrated successful T cell differentiation using the Notch ligand functionalized microbeads in both insert and coculture conditions with optimal results at a 1:1 bead to cell ratio. The Delta 1 transfected OP9 stromal cell system of T cell differentiation has been extensively used to demonstrate T cell commitment and development using human and murine progenitors from sources such as cord blood and bone marrow (de Pooter and Zuniga-Pflucker 2007; La Motte-Mohs et al., 2005; Schmitt et al., 2004). Immobilization of Notch ligand onto polystyrene coupled with supportive stroma is another method that has demonstrated effective T cell commitment and development in both murine and human progenitors (Dallas et al., 2005; Delaney et al., 2005; Ohishi et al., 2002). Our results perhaps most directly compare to studies performed by Hozumi and colleagues where fetal liver progenitors were transfected with intracellular Notch and incubated with supportive stroma (OP9) in both coculture and insert conditions (Hozumi et al., 2003). The differentiation efficiency for the insert

culture is comparable to Hozumi and colleagues results. The coculture differentiation efficiency may be lower due to the aggregation of beads, decreased cell to surface area ratio, decreased probability of bead-cell binding (with the addition of stroma present), as compared to insert culture. Further studies where higher cell numbers are utilized will most likely result in greater differentiation efficiencies for coculture conditions. When bead to cell ratio was increased to 5 to 1, a lower differentiation efficiency resulted. The aggregation of beads could have decreased effective ligand presentation but the increased bead number could have potentially become cytotoxic for cells. Cytotoxicity assays where effects of bead number on hematopoietic progenitors should be studied in the future. Something to note here is the fact that these studies were performed with DNA linker beads which have been shown to have low stability. Possible clustering of antibodies from cleaved DLL4 ligand aggregates has been mentioned in the previous section. Lefort and colleagues demonstrated that a very short exposure time to immobilized DLL4 is sufficient to commit human progenitors (Lefort et al., 2006). This result supports our findings. Also, although studies have found that continued Notch signaling is necessary for T cell differentiation in the thymus using OP9-DL1 cells, it is not necessary that these results apply to DLL4. Also, our level of assessment here is T cell commitment which may require a different level of Notch signaling. Future studies testing the effect of incubation with DLL4 functionalized microbeads will help to clarify the effects if any of incubation time.

6.1.4 Conclusions and future directions on scaffold properties and stromal cell coculture effects on hematopoietic differentiation of embryonic stem cells

In Chapter 6, we have successfully shown that scaffold properties can be optimized to induce increased hematopoietic differentiation efficiency of embryonic stem

cells. The ability to scale up the ex vivo production of hematopoietic progenitors for stem cell transplantation and even T cell differentiation applications (would require use of Notch ligands) is very beneficial and could potentially impact therapeutic applications. Low levels of hematopoietic differentiation from ESCs have plagued the field, however, prompting several studies in creative methods of improving differentiation efficiency (Dang et al., 2002; Dang et al., 2004; Gerecht-Nir et al., 2004; Gerecht-Nir et al., 2004b; Levenberg et al., 2003). Use of rotating bioreactors, scaffolds with pore sizes ranging from 50-500 μm, and use of capsules have all demonstrated improved differentiation and reduced agglomeration (what is thought to decrease differentiation efficency) (Dang et al., 2004; Gerecht-Nir et al., 2004; Gerecht-Nir et al., 2004b; Levenberg et al., 2003). We hypothesized that reduction of pore size would increase HPC differentiation. Immunophenotype analysis, where cell surface markers cKit and sca-1 were stained, revealed increased HPC cell numbers for the reduced pore size. Mechanical properties of scaffolds were also tested and shown to increase differentiation efficiency with increased elastic moduli. Finally, stromal cell coculture was shown to have a beneficial effect on differentiation efficiency, most likely due to secretion of supportive growth factors such as IL-7 and SCF. These results demonstrate that scaffold properties can be tailored to optimize the differentiation efficiency of stem cells. There are several concerns to be discussed, however. Scaffolds utilized in these studies consisted of poly lactic acid, a synthetic polymer that does not exist in the physiological environment but does degrade after time. Use of such a non-natural polymer elicits some concern for the generation of therapeutic cells. Future studies where biological components already existent in the bone marrow (site of hematopoiesis) could potentially give better differentiation efficiencies. Also, use of the scaffold platform can be problematic in cell retrieval and viability. Further studies improving cell retrieval and viability with gentler methods of

cell removal should be performed. And perhaps the greatest concern for such ex vivo differentiation studies is the realistic therapeutic impact the cells can make. Future studies where HPCs are implanted into mice and tested for reconstitution would elucidate the clinical application of such cells. Additional studies where HPCs are differentiated using established methods such as the OP9-DL1 cell line can also be conducted for ability to differentiate into various lineages.

6.1.5 Project design considerations

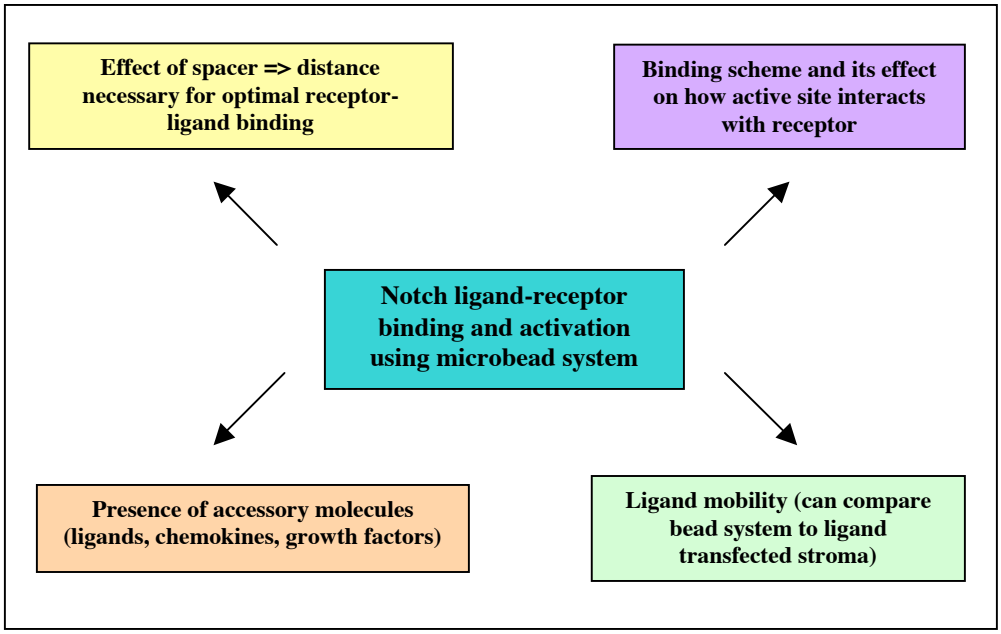
As evident from the discussion above, the current microbead system has not been fully characterized especially in terms of how it activates the Notch receptor. We have illustrated from characterization studies that varying ligand densities can be functionalized on the surface of the beads. We have also shown that the ligand stability is a serious concern with the DNA linker beads. Some amount of ligand release is also taking place with the beads without a DNA linker. How then Notch signaling is occurring when it does needs to be more fully studied. There are largely two possible outcomes of Notch ligand-receptor binding: successful Notch ligand-receptor binding and activation and Notch receptor engagement, resulting in the blocking of Notch signaling. It is very likely that the released DLL4 ligands are engaging the receptor and therefore preventing any subsequent Notch signaling from occurring (even with the addition of newly fabricated ligand beads.). Future studies are necessary to more closely examine the binding and activation events taking place.

It may be most beneficial to go back to the drawing board, so to speak, and really assess what the important properties of Notch ligand receptor binding are and how these properties can be applied to the microbead system. These properties have been summarized in **Figure 6.1**. For successful Notch ligand-receptor binding and thus Notch

receptor activation to take place, a variety of factors must be considered. The microbead system gives us the ability to test and study the effect of these factors in a systematic and quantitative manner. The binding scheme should obviously be simple and effective in functionalizing the ligand onto the receptor but should also be characterized in terms of how it affects the ligand-receptor binding. If, for example, a biotinylated DLL4 molecule was bound to streptavidin coated microbead, would the multiple DLL4 molecules on each streptavidin prevent the binding of any? Next, the effect of chain length on ligand-receptor binding should be characterized and considered. Chain length or spacer length is different from binding scheme. The introduction of a linear spacer imparts distance to the ligand from the microbead and can aid in the ligand-receptor binding. This can theroretically be another layer of control for the microbead system in terms of controlling ligand-receptor binding. Chain mobility is another important attribute of effective ligand-receptor binding. In the thymic microenvironment, for example, Notch receptors are expressed on developing T cell progenitors and Notch ligands are expressed on thymic stroma. Some level of mobility is likely for the cell surface ligands due to membrane diffusion and lateral mobility. This mobility most likely plays a role in receptor binding, potentially recruiting additional ligands or other molecules to increase the binding. Finally, additional accessory molecules may aid in the ligand receptor binding and should be explored with the microbead system.

The microbead system can be used not only as a technology for T cell production but as a tool for studying the receptor-ligand binding and Notch signaling cascade. By exploiting the ability to control the properties given above, we can better understand the Notch signaling process and perhaps extend the insights to other receptor-ligand binding systems, as well.

Figure 6.1 Important considerations in microbead design for Notch signaling applications.



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Appendix A

Influence of scaffold physical properties and stromal cell coculture on hematopoietic differentiation of mouse embryonic stem cells

1.1 INTRODUCTION

Embryonic stem cells (ESCs) are isolated from the inner cell mass of the blastocyst and have the ability to differentiate into all types of cells and self-renew indefinitely making them an attractive choice for *in vitro* and *in vivo* therapeutic applications (O'Shea 1999). Recently, ESCs have been shown to successfully differentiate into neural precursors, cardiomyocytes, hematopoietic cells in *in vitro* systems and exhibit functionality in animals (Johkura et al., 2003; Kaufman et al., 2001; Zhang et al., 2001). Hematopoietic studies using ESCs, however, have been primarily limited to 2D systems that have only limited ability to mimic the necessary cell–cell and cell–material interactions occurring in the three-dimensional (3D) in vivo microenvironment. As the prospect for stem cell based tissue regeneration increases, the development and thorough characterization of 3D based stem cell differentiation systems is becoming increasingly crucial.

The site of hematopoiesis migrates in a developing embryo from the yolk sac and aorta-gonad-mesonephros region to the bone marrow where hematopoiesis occurs in adults. During hematopoiesis, cell-mediated and soluble growth factors bind to cell surface proteins to trigger or inhibit signal cascades, committing stem cells to either a myeloid or lymphoid lineage. The meshwork of stroma, ECM, and bone that make up the microenvironment provide not only the physical framework for cell proliferation and differentiation, but are also intimately involved in adhesion, cytokine presentation, and

cell growth (Goldsby 2003; Mantalaris et al., 1998; Nilsson et al., 1998; Sternberg 1997; Vituri et al., 2000). *In vitro* hematopoietic differentiation systems exploit these cell–stroma interactions to drive embryonic stem cells to hematopoietic lineages.

Scaffolds are increasingly being applied to stem cell cultures in order to more closely resemble the 3D *in vivo* microenvironment (Bagley et al., 1999; Gerecht-Nir et al., 2004; Levenberg et al., 2003; Sasaki et al., 2002; Sasaki et al., 2003). Scaffolds offer the physical support that enable cell–cell and cell–material interactions facilitating a more tissue-like structure (Sasaki et al., 2002). For example, an *ex vivo* thymic microenvironment in a tantalum-based scaffold was shown to result in efficient differentiation of human stem cells into T cells while human ESCs were shown to differentiate into vasculature-like structures in PLGA-based scaffolds (Levenberg et al., 2003; Poznansky et al., 2000). Scaffold based studies have particularly been instrumental in understanding embryoid body (EB) formation and hematopoiesis. Studies involving stem cell encapsulation in alginate capsules and stem cell culture in alginate-based scaffolds, for example, have elucidated the role of inhibiting EB agglomeration in increasing hematopoietic differentiation efficiency (Dang et al., 2004; Gerecht-Nir et al., 2004). However, systematic assessment of the role of scaffold physical properties and culture microenvironment, e.g. influence of stromal cells, on the efficiency of ESC hematopoiesis has not been reported.

We recently demonstrated the advantageous effects of 3D scaffold culture and dynamic culture conditions on hematopoietic differentiation of mouse ESCs (Liu and Roy 2005). The 3D porous substrate coupled with dynamic flow conditions limited EB aggregation and increased hematopoietic progenitor frequency in differentiated ESCs in a highly reproducible manner. Our objective for this study was to systematically investigate the microenvironmental effects of 3D scaffold culture on ESC differentiation into

hematopoietic progenitors. Previous studies have demonstrated the significance of EB confinement as a way of optimizing hematopoietic progenitor cells (HPCs) generation (Dang et al., 2004; Gerecht-Nir et al., 2004). Mechanical properties have also been shown to affect ESC differentiation in 3D structures (Battista et al., 2005; Levenberg et al., 2003).

Poly(L-lactic acid) (PLLA) based scaffolds offer the advantage of highly tunable mechanical and physical properties that can be tailored for individual applications (Ishaug-Riley et al., 1998). In this study, we varied the pore size and polymer composition of the scaffolds and explored their effect on hematopoietic differentiation of ESCs. Stromal cells such as OP9 have been widely used in hematopoietic applications due to their ability to secrete relevant growth factors (Cho et al., 1999; Nakano et al., 1994; Nakano 1996; Vodyanik et al., 2005). Here we evaluated (a) how coculture of ESCs with bone marrow derived stromal cells (OP9) enhances the efficacy of HPC generation and (b) whether direct cell–cell contact between ESC and OP9 cells is necessary for this enhanced differentiation. Our results indicate that paracrine signaling from OP9 cells can increase ESC hematopoiesis in scaffold cultures. Such a systematic study could provide critical insights in optimizing culture conditions and ultimately offer the potential to produce hematopoietic progenitors from ESCs in a scalable manner for therapeutic applications.

1.2 MATERIALS AND METHODS

1.2.1 Scaffold fabrication

PLLA scaffolds were synthesized using the standard salt leaching procedures, as described by Ishaug-Riley et al. (Ishaug-Riley et al., 1998). Briefly, PLLA (Medisorb,

Low IV, Alkermes, Cambridge, MA) was dissolved in dichloromethane (7.5%, 10% and 20% w/v) and 4.5 g of varying sizes of NaCl particles (sieved to <150, 150–425 or 425< mm) was added. The PLLA/NaCl mixture was poured into a 6 cm glass Petri dish, followed by solvent evaporation and extensive salt leaching in dH2O. Scaffolds were cut into 8 mm diameter constructs using a biopsy punch following solvent evaporation. Scaffolds were then freeze-dried for 48 h and stored under vacuum in the desiccator until further use.

1.2.2 ESC culture, maintenance and differentiation

Undifferentiated mouse R1 ESCs (a gift from A. Nagy, Mount Sinai Hospital, Ontario, Canada) were expanded on leukemia inhibiting factor (LIF)-producing irradiation-inactivated embryonic fibroblast cells (STO cells, Shan Maika, Austin, UT) for 10 days in complete DMEM medium (Invitrogen, Carlsbad, CA) containing 20% fetal bovine serum (Hyclone, Logan, UT), 2 mM L-glutamine, 1 mM sodium pyruvate, 0.1 mM non- essential amino acids, 5 x 10^{-5} M 2-mercaptoethanol, penicillin G (100 U/mL), and streptomycin (10 μg/mL) (all from Invitrogen, Carlsbad, CA). This expansion process on LIF maintains the cells in their undifferentiated state (Williams et al., 1988). At this stage, 1 x 10^5 cells (unless otherwise stated) were seeded dropwise on sterilized PLLA scaffolds (7.5%, 10%, and 20% w/v, 150–425 μm pore size or <150, 150–425 or 425< μm, 10% w/v PLLA) for 7 h at a density of 2 x 10^6 cells/mL. The scaffolds were sterilized in 70% ethanol for 1 h prior to seeding. Trapped air in scaffolds was removed as described by Ma et al. (Ma et al., 2003). After 7 hr incubation at 37ºC, unseeded cells in supernatant from each scaffold were quantified to measure seeding efficiency. A seeding efficiency of 75–90% was consistently achieved using this process.

For cell seeding studies, 10^4, 5 x 10^4, 10^5, 2 x 10^5, and 5 x 10^5 undifferentiated ESCs were seeded in scaffolds (10% w/v PLLA, 150–425 μm pore size) as described above. Seeded cells were cultured in 24 well tissue culture plates in static culture for 7 or 14 days. The differentiation medium, consisting of αMEM (Invitrogen, Carlsbad, CA), 20% FBS (Hyclone, Logan, UT), 2.2 g/L sodium bicarbonate (Invitrogen, Carlsbad, CA), penicillin G (100 U/mL), and streptomycin (10 μg/mL) (Invitrogen, Carlsbad, CA) was replaced every 4–5 days. For cell removal, scaffolds were first rinsed in Dulbecco's phosphate-buffered saline (D-PBS) (Invitrogen, Carlsbad, CA), followed by incubation with either trypsin or Accumax (Innovative Cell Technologies, San Diego, CA) in a 37°C water bath for 10 min, and finally vortexed and washed gently using serum- containing medium to detach the cells.

1.2.3 OP9 cell coculture

OP9 cells, a mouse bone marrow derived stromal cell line, were used to study the effect of stromal cell coculture on hematopoietic differentiation from ESCs. These cells (generous gift from Tammy Reid, Stanford Lab, University of Toronto, Toronto, Canada) were maintained in differentiating medium described above. Unless otherwise stated, scaffolds fabricated using a 10% w/v PLLA solution with pore sizes of 150–425 μm were used for this study. All coculture conditions were designed to study the paracrine effects of the stromal cells, thus avoiding direct ESC–stromal cell contact. For cocultures with R1 cells in 3D scaffolds and OP9 cells in 2D plates (3DR1/2DOP9), 3 x 10^4 OP9 cells were seeded onto wells in 24 well tissue culture-treated polystyrene plates while R1 cells were separately seeded on 8 mm diameter scaffolds. After cell attachment, scaffolds were placed on OP9 cell layers. It is conceivable that this design could result in some limited contact between the OP9 cells on the plate and few ESCs at the very bottom of the

scaffolds. However, for majority of the ESCs in the scaffold would not have any stromal cell contact. For cocultures with both R1 and OP9 cells in 3D scaffolds (3DR1/3DOP9), 3 x 10^4 OP9 cells were seeded onto PLLA scaffolds of 3 mm diameter. R1 cells were seeded on separate 8 mm diameter scaffolds as described above. Both scaffolds were *placed together* in 24 well plates. Care was taken to avoid physical contact between the scaffolds. Difference in scaffold size was used to distinguish the ESC scaffolds from the stromal cell scaffolds. For 3DR1 controls, R1 cells were seeded onto scaffolds without a stromal cell layer. For the 3DR1/3DOP9 condition, new scaffolds, seeded with fresh OP9 cells were added to the wells every four days along with medium change. For the 3DR1/2DOP9 condition, ESCs were re-seeded on fresh OP9 cell layers at the same time points. Stromal coculture was performed for 2 weeks followed by flow cytometry analysis.

1.2.4 Flow cytometry analysis

Flow cytometry was performed similar to methods described previously in Liu and Roy (Liu and Roy 2005). Generation of HPCs was assessed through staining of HPC-specific cell surface markers on days 7 and 14. Cells were washed twice in FACS buffer, 1% bovine serum albumin (Sigma-Aldrich, St. Louis, MO) and 0.05% sodium azide (Sigma-Aldrich, St. Louis, MO) in PBS before staining and blocked for non-specific binding using anti-mouse CD16/CD32 Fc Block (BD Pharmingen, San Diego, CA) for 10 min at 4°C. Staining with antibodies against specific hematopoietic markers was performed at concentrations of 1 μg/100 μl of FACS buffer and washed prior to staining with secondary fluorescently labeled antibodies, if necessary. Isotype controls (eBioscience, San Diego, CA) were used as negative controls. All cells were washed twice in FACS buffer and suspended in fresh buffer before flow cytometry analysis using

FACSCalibur (Becton Dickinson, San Diego, CA) and CellQuest 3.1 software (BD Biosciences, San Jose, CA). Fluorescently labeled antibodies against cKit and sca-1 (cKit-PE and sca-1-FITC, BD Pharmingen, San Diego, CA) were used for the identification of ESC-derived HPCs. Only cells that stained for both cKit and sca-1 were identified as HPCs. Simultaneous expression of cKit and sca-1 (dual staining) surface markers is a common hallmark of hematopoietic stem cells in mice (Wognum et al., 2003).

1.2.5 Compressive modulus testing

Mechanical properties of scaffolds (7.5%, 10%, and 20% w/v, 150–425 μm pore size and 10% w/v <150, 150–425, 425< μm pore size) were characterized by testing four specimens from each scaffold type under compression between parallel plates using an Instron (In-Spec 2200 Benchtop Tester, Instron Corporation, Canton, MA) and In-SpecTM 2200 PDA Emulator software (Instron Corporation, Canton, MA). A 125 N compression static load cell and 0.2 mm/s cross-head speed were used. Young's modulus for each sample was calculated by performing a regression on the linear region of the load-compression data.

1.2.6 Statistical analysis

All experiments were performed in triplicate except for pore size studies (performed in duplicate). Analysis of statistical significance was performed using a one tailed Student's *t*-test. Significance was determined using a 95% probability value ($p<0.05$).

1.3 RESULTS

Recently we reported the effects of 3D scaffold-based culture on hematopoietic differentiation of mouse ESCs (Liu and Roy 2005). Our results indicated hematopoietic differentiation efficiency in 3D systems over 2D tissue culture plates. Here, we further explored the effects of specific 3D culture conditions, specifically cell seeding density, scaffold properties and stromal cell coculture on hematopoietic differentiation. The experimental variables studied are summarized in **Table 1.1**. PLLA-based scaffolds were selected as 3D substrates due to their ease of fabrication, highly tunable physical and mechanical properties and their widespread application in tissue engineering. In addition, selection of PLLA ensured minimal to no degradation of the scaffold during the period (one week) of the differentiation experiments. Representative scaffolds were routinely evaluated by scanning electron microscopy, which indicated interconnecting pore structure and uniform pore sizes (data not shown).

1.3.1 Higher cell seeding density results in increased HPC differentiation

Seeding density is known to affect stem cell differentiation and can be optimized to result in higher differentiation efficiencies (Gerecht-Nir et al., 2004; Poznansky et al., 2000; Tsai et al., 2000; Wilson et al., 2002). To study the effect of cell density on hematopoietic differentiation, we seeded ESCs at 10^4, 5 x 10^4, 10^5, 2 x 10^5, and 5 x 10^5 cells per scaffold (10% w/v PLLA, 150–425 μm, 8 mm diameter) and cultured the cells for 7 days as described above. Cells were then harvested from scaffolds, stained for both cKit and sca-1 expression and analyzed using flow cytometry. As shown in **Figure 1.1**, HPC generation appears to increase with increasing cell seeding density in the range investigated. Statistical analysis using Student's *t*-test indicated a significant difference between 10^4 cells/scaffold, 10^5, and 5 x 10^5 cells/scaffold ($p<0.05$).

1.3.2. HPC generation is impaired at larger pore sizes

Porous structures such as PLLA scaffolds provide a physical framework and enable cell ingression and nutrient diffusion for tissue engineering applications. To evaluate how scaffold pore sizes influence ESC hematopoiesis, 10% w/v PLLA scaffolds with three different pore size ranges were seeded with 10^5 ESCs and cultured for one week followed by flow cytometry analysis for HPC generation. Pore sizes of <150 μm, 150–425 μm and >425 μm were produced using sieved salt particles during scaffold fabrication. As shown in **Figure 1.2A**, HPC generation decreases with increased pore size. The smallest pore size (<150 μm) yielded significantly more (1.68 and 2.06 fold) HPCs when compared to the larger pore sizes.

To address any changes in mechanical properties due to pore size differences, we measured the compressive moduli of the scaffolds. Static compressive loads of 125 N were applied using an Instron (In-Spec 2200 Benchtop Tester) at 0.2 mm/s on 10% PLLA scaffolds with various pore sizes until failure. Compressive modulus varied slightly between the smaller pore sizes and decreased significantly ($p<0.05$) for pore size greater than 425 μm, as indicated in **Figure 1.2B**. Statistical analysis indicated a significant difference in HPC generation between all scaffolds tested.

1.3.3. HPC generation increases with scaffold polymer concentration, compressive modulus

To investigate the effect of mechanical properties on HPC generation, we fabricated PLLA scaffolds with three different polymer concentrations. Scaffolds consisting of 7.5%, 10% and 20% (w/v) PLLA with pore sizes of 150–425 μm were seeded with 10^5 ESCs and cultured for a week. **Figure 1.3A** indicates that, on average,

HPC generation increases with increasing polymer concentration in the range investigated. Statistical analysis using Student's *t*-test showed a significant difference between 7.5% and 20% PLLA scaffolds.

Scaffold mechanical properties have also been shown to affect cell differentiation and can be modified by changing the polymer concentration (Battista et al., 2005; Levenberg et al., 2003). To measure the compressive moduli of the scaffolds with varying PLLA concentration, a 125 N static compressive load was applied using an Instron (In-Spec 2200 Benchtop Tester) at 0.2 mm/s on 7.5%, 10% and 20% PLLA scaffolds (with 150–425 μm pore sizes) until failure. As shown in **Figure 1.3B**, the compressive moduli increases with polymer percentage and range from 20.29±2.45 to 25.72±3.86 MPa for 7.5% and 20% PLLA scaffolds, respectively.

1.3.4. Paracrine coculture with stromal cells significantly enhances HPC generation

Stromal cells such as OP9 are often used in hematopoiesis due to their inherent ability to secrete relevant growth factors such as interleukin-7 (IL-7) and stem cell factor (SCF) (Cho et al., 1999). However, most of these studies were performed using a mixed coculture system. Here, we explored the ability of paracrine signals (i.e. no cell–cell contact) from OP9 mouse stromal cells to improve HPC generation from ESCs cultured in polymer scaffolds. Briefly, we seeded 3 x 10^4 OP9 cells on 3 mm diameter 10% PLLA scaffolds with pore sizes of 150–425 μm or individual wells in a tissue culture-treated 24 well plate. Next, we seeded 10^5 ESCs on 10% (w/v) PLLA scaffolds with pore sizes of 150–425 μm and placed these scaffolds onto a OP9 layer in well (3DR1/2DOP9) or placed the scaffolds into a well along with OP9 cells seeded on a separate scaffold (3DR1/3DOP9). 10^5 ESCs seeded on 10% (w/v) PLLA scaffolds with pore sizes of 150–425 μm without OP9 support were used as controls. After two weeks of culture,

cells were harvested, stained, and analyzed using flow cytometry for dual expression of cKit and sca-1. As shown in **Figure 1.4**, our results indicate a significant increase in hematopoiesis in the presence of OP9 cells ($p<0.05$, Student's *t*-test).

1.4 DISCUSSION

In our previous work, we demonstrated the beneficial effects of 3D scaffolds on hematopoiesis using the commercially available Cytomatrix™ system. This study prompted us to further explore the effects of physical and mechanical properties of 3D substrates on HPC generation. Several recent studies have provided tremendous insight into EB formation and differentiation processes both in 2D and 3D culture systems. Dang and colleagues illustrated the importance of EB aggregation and cell attachment, specifically, in providing cell fate decisions during hematopoiesis using liquid suspension, methylcellulose culture, hanging drop and attached culture in the murine system. A low cell seeding density was found to result in most efficient EB formation due to the maximum size limitation (Dang et al., 2002). In a related study, Gerecht-Nir and colleagues utilized a rotating bioreactor system with hydrophilic alginate scaffolds with pore sizes of 50–200 μm to study human EB formation in a physically constricted environment. EB agglomeration was inhibited in both rotating and scaffold microenvironments when compared to static and petri dishes, respectively. Seeding density was not found to affect EB formation in scaffolds (Gerecht-Nir et al., 2004). Levenberg et al. suggested the importance of mechanical properties in supporting human ESC growth and organization in a matrigel/fibronectin coated PLGA/PLLA scaffolds (pore sizes of 250–500 μm) culture system (Levenberg et al., 2004). Finally, semi-interpenetrating polymer networks of collagen, fibronectin and laminin were shown to

influence ESC differentiation into specific cell lineages. Mechanical properties, such as the elastic modulus was shown to inhibit EB growth and differentiation when increased from 16 to 34 Pa (Battista et al., 2005).

Our goal was to systematically study HPC generation in 3D PLLA scaffold structures by varying the scaffold physical and mechanical properties as well as by changing the cell microenvironment through cell seeding density and coculture conditions. We used flow cytometry analysis of murine HPC markers (dual expression of cKit and sca-1) to evaluate the effect of pore size, polymer composition, compressive moduli, seeding density and stromal cell coculture on hematopoiesis.

Although previous studies suggested no difference in EB formation and HPC generation for various seeding densities, our results indicate that higher ESC seeding densities in PLLA scaffolds could result in significantly higher HPC generation (Dang et al., 2002; Gerecht-Nir et al., 2004). This could be a result of specific scaffold material (PLLA) or a factor of the cell density range evaluated.

Larger pore sizes were found to hinder HPC generation. Recent reports have emphasized the importance of confining EBs to prevent agglomeration. These studies utilized scaffold pore sizes or capsule sizes of 50–200 μm and 100–150 μm to limit EB aggregation (Dang et al., 2002; Gerecht-Nir et al., 2004). Our results indicated pore sizes of less than 150 μm to be the most beneficial for HPC generation, which is consistent with these studies.

Polymer percentage and mechanical properties also seem to play a role in dictating HPC generation efficiency (Battista et al., 2005). We found that substrate scaffolds with higher modulus were more conducive towards HPC generation. Battista and colleagues, however, reported an inhibitory effect on EB formation and differentiation when the elastic modulus was raised from 16 to 34 Pa in semi-

interpenetrating network scaffolds of collagen, fibronectin and laminin (Battista et al., 2005). Our compressive moduli, however, were on a scale of MPa. Changes in mechanical moduli on such different scales may affect EB formation differently. It is also possible that the substrate material itself could have an influence on how mechanical properties affect ESC differentiation.

Finally, we observed a significant improvement in HPC generation with OP9 stromal cell coculture. Recent studies by Thomson and colleagues have demonstrated up to 20% HPC differentiation efficiency of human ESCs under OP9 coculture conditions (Vodyanik et al., 2005). Here, we obtained similar results using scaffold-cultured mouse R1 ESCs and OP9 cells either seeded on scaffolds or on tissue culture plates. These results suggest that, direct cell–cell contact may not be a necessary condition for the increased hematopoiesis induced by OP9 cells. Soluble factors such as IL-7 and SCF secreted by OP9 cells might provide sufficient cues for HPC generation.

In conclusion, we have demonstrated that scaffold properties and culture conditions significantly affect hematopoiesis of mouse ESCs. Smaller scaffold pore size, increase mechanical stiffness, coculture with stromal cells and increasing cell seeding density were all shown to increase HPC generation. These results suggest a critical need for microenvironment optimization for efficient differentiation of ESCs in 3D scaffold-based cultures.

Table 1.1 Summary of Various Culture Conditions

Culture Parameters	*Description*
Scaffold properties	
Pore size	<150_m, 150-425_m, 425_m<
Polymer percentage	7.5%, 10%, 20% w/v
Seeding density	10^4, $5x10^4$, 10^5, $2x10^5$, $5x10^5$ cells/scaffold
Stromal cell coculture	
3D	ES cells seeded on 3D scaffold
3D-2D	ES cells seeded on 3D scaffold; scaffold placed on OP9 cell layer cultured on tissue culture plates
3D-3D	ES cells seeded on 3D scaffold; OP9 cells seeded on separate scaffold and cultured together on a single well

Figure 1.1 Effect of cell seeding density on HPC generation from ESCs in PLLA scaffolds. ESCs were cultured at various seeding densities on 10% w/v PLLA scaffolds with pore sizes of 150–425 μm for 1 week before harvesting, staining for cKit and sca-1 expression and analysis using flow cytometry. Dual expression of cKit and sca-1 represents HPC population. (A) Flow cytometry data. Numbers in quadrant correspond to cell percentages. Upper right quadrant indicates HPCs expressing both cKit and sca-1. (B) Bar graph showing percentage of HPC generated for different seeding densities (n = 3). *indicates $p<0.05$ compared to the lowest seeding density using a Student's *t*-test.

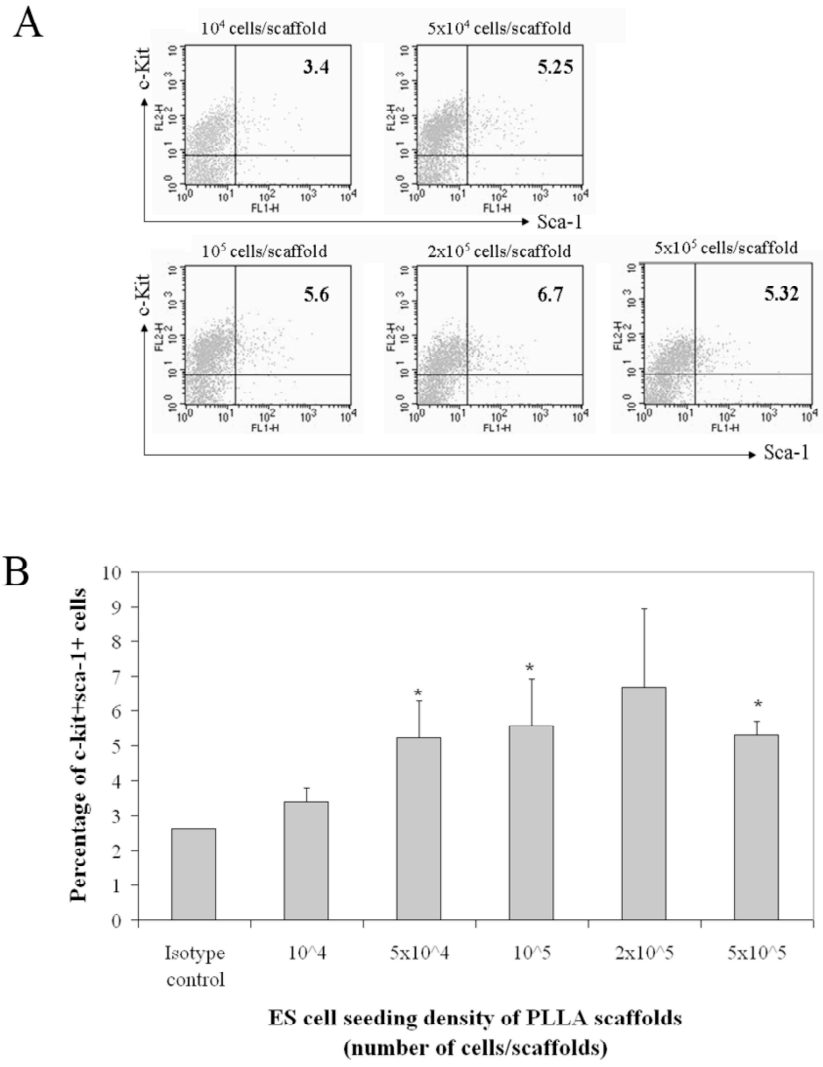


Figure 1.2 Effect of pore size on HPC generation from ESCs in PLLA scaffolds. HPC generation is impaired at larger scaffold pore sizes. 10^5 ESCs were cultured on 10% w/v PLLA scaffolds with various pore sizes for 1 week followed by harvesting, staining for cKit and sca-1 expression and analysis using flow cytometry. Dual expression of cKit and sca-1 represents HPC population. (A) Flow cytometry data. Numbers in each quadrant correspond to cell percentages. Upper right quadrant indicates HPCs expressing both cKit and sca-1. (B) Bar graph demonstrating HPC generation decreases with larger scaffold pore sizes. Percentage of cells expressing both cKit and sca-1 at various pore sizes (closed squares) are simultaneously plotted with the corresponding scaffold compression moduli (closed circles). Experiments were performed in duplicate. *indicates groups with significant difference in HPC generation as compared to pore size of <150 μm ($p<0.05$, Student's *t* test). ** indicates group with significant difference in HPC generation as compared to pore size of 150–425 μm ($p<0.05$, Student's *t*-test). #, # # indicates significant difference in compression modulus as compared to scaffolds with pore size < 150 μm, and 150–425 μm ($p<0.05$, Student's *t*-test).

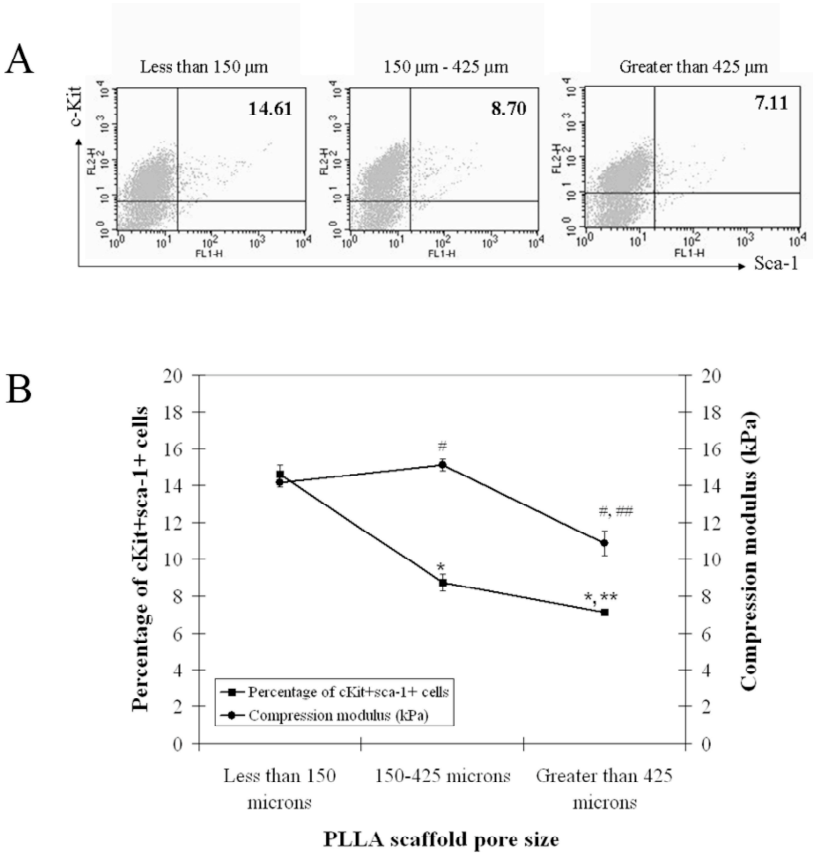


Figure 1.3 Effect of scaffold polymer concentration on HPC generation from ESCs. 10^5 ESCs were cultured on PLLA scaffolds fabricated using various polymer concentrations followed by cell harvesting, staining for cKit and sca-1 expression and analysis using flow cytometry. All scaffolds had pore sizes of 150–425 μm. Dual expression of cKit and sca-1 represents HPC population. (A) Flow cytometry analysis. Numbers in each quadrant correspond to cell percentages. Upper right quadrant indicates HPCs expressing both cKit and sca-1. (B) HPC generation may correlate with mechanical properties of scaffold. cKit and sca-1 expression at various polymer concentrations (closed squares) are simultaneously plotted with corresponding scaffold compression moduli (closed circles). *indicates p value of 0.05 when compared to 7.5% w/v PLLA scaffold using Student's *t*-test. ** indicates group with significant difference in HPC generation as compared to 7.5% w/v PLLA scaffold ($p<0.05$, Student's *t*-test).

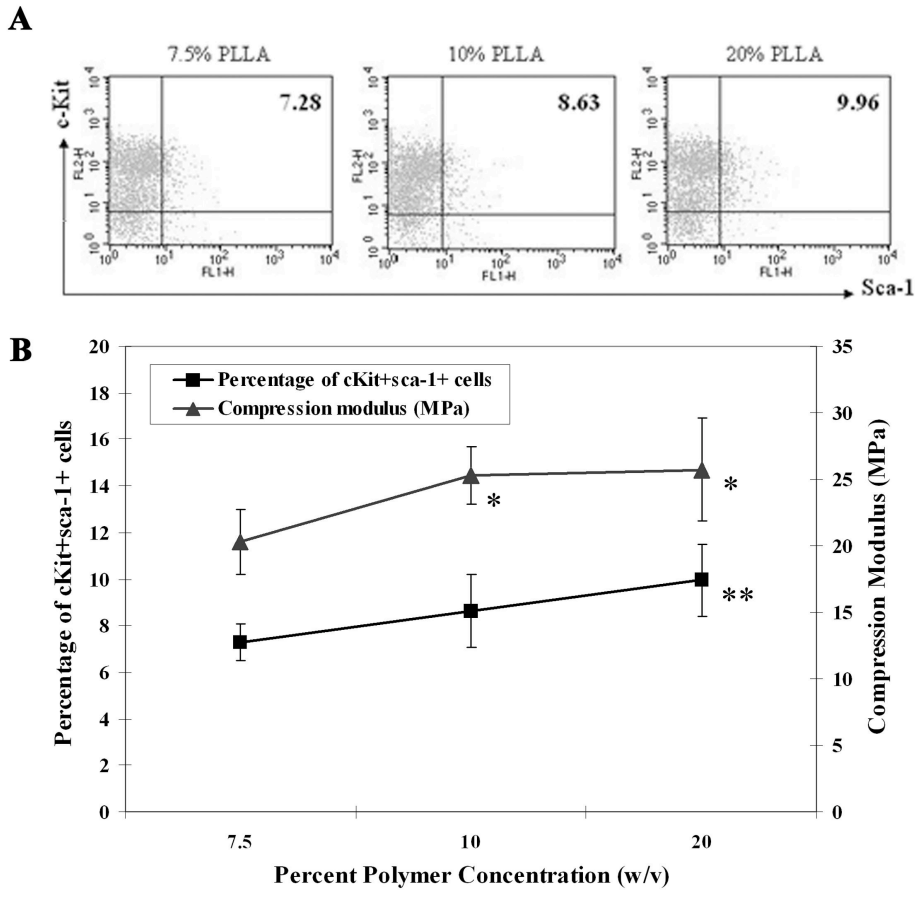
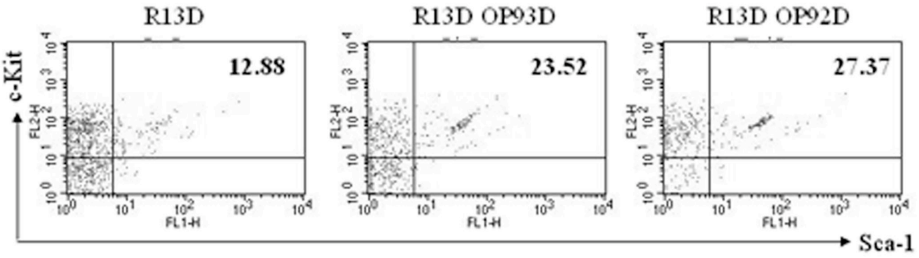
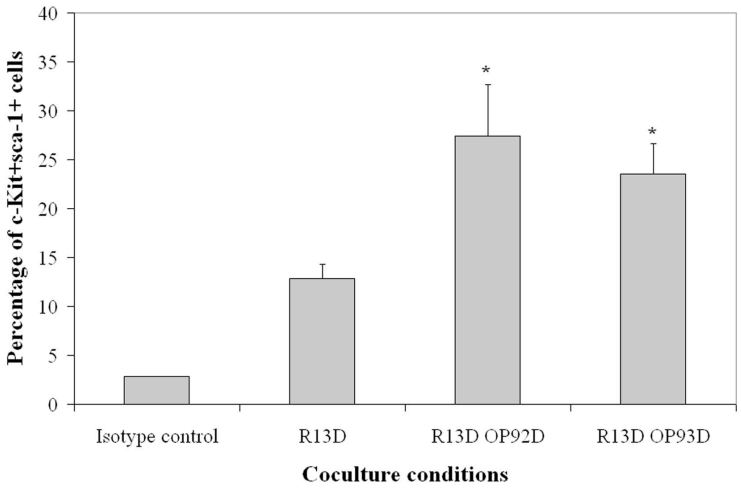


Figure 1.4 Effect of stromal cell coculture on HPC generation from ESCs in PLLAscaffolds. Coculture with OP9 cells may enhance HPC generation from R1 ESCs. 10^5 ESCs were cultured on 10% w/v PLLA scaffolds with pore sizes of 150–425 μm for 2 weeks followed by staining for cKit and sca-1 expression and flow cytometry analysis. Dual expression of cKit and sca-1 represents HPC population. (A) Flow cytometry analysis. Numbers in quadrant correspond to cell percentages. Upper right quadrant indicates HPCs expressing both cKit and sca-1. (B) Bars indicate percentage of HPC generated under each culture conditions. All experiments were performed in triplicate. *indicates a $p<0.05$ compared to the lowest polymer concentration using a Student's *t*-test.

A



B



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APPENDIX B

Important Protocols

Created by: Sabia Taqvi
Revised: May 15, 2007

Immunofluorescence detection of antiHIS coating on beads (flow cytometry)

MATERIALS

CELLection biotin binder kit (Invitrogen, 4ºC)
Biotinylated antiHIS antibody (R&D systems, -20ºC)
Washing buffer (PBS, 0.1%BSA, 0.05% sodium azide, 4ºC)
FACS staining buffer (PBS, 1% BSA, 0.05% sodium azide, 4ºC)
Blocking buffer (PBS, 3%BSA, 4ºC)
Goat anti-mouse IgG, FITC conjugate (Calbiochem, -20ºC)
Streptavidin-PE (BDBiosciences, 4ºC)
FACS tubes
Lo protein binding tubes (0.65ml, smaller sizes if available)
Dynalbiotech magnet
0.1-10ul, 2-20ul and 100-1000ul pipettors with tips
Rotator
Foil

METHODS

Dynabeads Washing Procedure

1. Resuspend the Dynabeads thoroughly and transfer the desired amount of beads (2 μl) to a tube suitable for the Dynal MPC.
2. Place the tube in Dynal MPC for 1 minute. Make sure the solution is clear and pipette off the fluid without disturbing the beads.
3. Remove the tube from the Dynal MPC, add 1 - 2 ml buffer (e.g. PBS with 0.1% BSA) and resuspend. If using smaller sized tubes, use 0.5 ml.
4. Place the test tube in the Dynal MPC for 1 minute, remove the buffer and take the tube out of the Dynal MPC.
5. Resuspend the washed Dynabeads back in 2 μl buffer ($4x10^8$ beads/ml).

Dynabeads Coating Procedure

1. We will be coating in a volume of 10 μl to avoid errors with low volumes. Thus, add the calculated amount of total buffer (subtracting 1μl for both the beads and antiHIS antibody) and add this to the washed bead suspension. Resuspend washed Dynabeads well by thoroughly pipetting.
2. Prepare antiHIS antibody solutions in 0.1% BSA in PBS with varying concentrations in low protein binding tubes. Make at least 3-5 μl of each to avoid running out of solution. Calculate concentrations such that 1 μl is the volume added to each tube.

3. Add 1 μl of each concentration to each tube and mix well. Pipette carefully and only dispense liquid directly into bottom of tube.
4. Rotate the bead and antibody suspension for 30 minutes at room temperature.
5. Add 0.5/1ml PBS with 0.1% BSA to each tube and mix well.
6. Place the tube in a Dynal MPC for 1 minute. Make sure the solution is clear and pipette off the fluid.
7. Remove the tube from the Dynal MPC and add 0.5/1ml PBS with 0.1% BSA.
8. Repeat steps 5-7 two times. Aspirate out all volume completely.

Dynabeads Staining Procedure

1. *Blocking* - Add 100 μl of 3% BSA in PBS and incubate for ≥30 min at 4ºC.
2. Place samples in Dynal MPC for 1 minute and remove supernatant.
3. *Antibody incubation* – ***In DARK***. Add FITC goat anti-mouse antibody to each tube at a concentration of 0.25 μg/1million beads in a staining volume of 100 μl and incubate for 30 min at 4ºC on rotator.
4. *Washing* - ***In DARK***. Wash twice with 500 μl of FACS buffer.
5. ***In DARK***. Aspirate off supernatant completely. Resuspend in 250μl of FACS buffer and add to prelabeled FACS tubes.
6. Cover FACS tubes in Styrofoam racks with paraffin and foil and proceed to FACS. Place racks in large foam box for convenience. Remember to take thumb drive, notebook with FACS settings, key and access card and of course samples. Proceed to FACS machine.

**For studies with streptavidin-PE, a concentration of 0.25 μg/1million beads was used as well.

Created by: Sabia Taqvi
Revised: May 15, 2007

Immunofluorescence detection of DLL4 coating on beads (flow cytometry)

MATERIALS

CELLection biotin binder kit (Invitrogen, 4ºC), *concentration: 4x10^8 beads/ml*
Biotinylated antiHIS antibody (R&D systems, -20ºC), *concentration: 0.25 mg/ml*
Recombinant DLL4 (R&D systems, -20ºC), *concentration: 0.25 mg/ml*
Washing buffer (PBS, 0.1% BSA, 0.05% sodium azide, 4ºC)
FACS staining buffer (PBS, 1% BSA, 0.05% sodium azide, 4ºC)
Blocking buffer (PBS, 3% BSA, 4ºC)
Rat anti-mouse monoclonal DLL4 antibody (R&D systems, -20ºC), *concentration: 0.5 mg/ml*
Fluorescein isothiocyanate (FITC) mouse antirat IgG (H+L) (R&D systems, -20ºC), *concentration: 0.5 mg/ml*
Anti-rat quantum simply cellular kit (Bangs labs, 4ºC)
FACS tubes
Lo protein binding tubes (0.65ml, smaller sizes if available)
Dynalbiotech magnet
0.1-10μl, 2-20μl and 100-1000μl pipettors with tips
Rotator
Foil

METHODS

Dynabeads Washing Procedure (all performed sterilely)

6. Keep all reagents cold if possible.
7. Resuspend the Dynabeads thoroughly and transfer the desired amount of beads (25 μl is the common amount made) to a tube suitable for the Dynal MPC.
8. Place the tube in Dynal MPC for 1 minute. Make sure the solution is clear and pipette off the fluid without disturbing the beads.
9. Remove the tube from the Dynal MPC, add 1 ml buffer (e.g. PBS with 0.1% BSA) and resuspend. If using smaller sized tubes, use 0.5 ml.
10. Place the test tube in the Dynal MPC for 1 minute, remove the buffer entirely and take the tube out of the Dynal MPC.
11. Resuspend the washed Dynabeads back in 25 μl buffer ($4x10^8$ beads/ml).

Dynabeads Coating Procedure (all performed sterilely)

9. Prepare antiHIS antibody solutions in 0.1% BSA in PBS with calculations of antiHIS antibody at 100 ng/10^7 beads. For example, for 25 μl or 10^7 beads,

you need 100ng of antibody + beads + buffer = 25 μl total volume. Adjust antibody concentration so that total 25 μl is not exceeded.

10. Remove buffer from washed beads, add antibody and fresh buffer so that total volume is 25 μl or whatever original bead volume was.
11. Rotate the bead and antibody suspension for 30 minutes at room temperature.
12. Add 0.5/1ml PBS with 0.1% BSA to each tube and mix well.
13. Place the tube in a Dynal MPC for 1 minute. Make sure the solution is clear and pipette off the fluid.
14. Remove the tube from the Dynal MPC and add 0.5/1ml PBS with 0.1% BSA.
15. Repeat steps 5-6 two times. Aspirate out all volume completely.
16. Prepare DLL4 solutions in 0.1% BSA in PBS in low protein binding tubes. For 25 μl and 10ng/10^7 beads, you will need 10 ng of DLL4. Make sure the volume does not exceed 25 μl. Prepare all concentrations in the same way.
17. Add DLL4 to antibody coated beads and add buffer so that volume totals out to be 25 μl.
18. Rotate the antibody coated bead and DLL4 suspension for 30 minutes at room temperature.
19. Add 0.5/1ml PBS with 0.1% BSA to each tube and mix well.
20. Place the tube in a Dynal MPC for 1 minute. Make sure the solution is clear and pipette off the fluid.
21. Remove the tube from the Dynal MPC and add 0.5/1ml PBS with 0.1% BSA.
22. Repeat steps 5-6 two times. Aspirate out all volume completely.
23. Resuspend in 25 μl of fresh buffer and proceed with staining.

Dynabeads Staining Procedure (can be done benchtop)

7. For primary antibody solution, you want to add 2-4 μg/million beads of anti-DLL4 antibody (concentration taken from datasheet) into FACS buffer. For the secondary antibody solution, you want to add 0.25 μg/million beads of FITC anti-rat IgG (concentration taken from datasheet) into FACS buffer. For the QC beads, you want to add 50% of amount of antibody for 4 million cells per sample. Also, when you make the antibody solution, you want the total staining volume to be 100 μl. Also label FACS tubes at this point.
8. *Blocking* - Remove buffer from tubes and add 100 μl of 3% BSA in PBS and incubate for ≥30 min at 4ºC.
9. Place samples in Dynal MPC for 1 minute and remove supernatant.
10. *Primary antibody incubation* – Add 100 μl of primary antibody solution to samples, tap several times and incubate for 30 min at 4ºC.
11. *Washing* - Add 500 μl of FACS buffer and pipette up and down several times. Place in magnet, aspirate out supernatant and repeat buffer washing. Aspirate out supernatant completely. For QSC kit, you will need to add 500 μl and centrifuge at 4ºC 2500 rpm for 5 min. Remove supernatant and repeat.

12. **IN DARK** *Secondary antibody incubation* – Add 100 μl of secondary antibody solution to samples, tap several times and incubate for 30 min at 4ºC in 100 μl volume.
13. **IN DARK** *Washing* - Add 500 μl of FACS buffer and pipette up and down several times. Place in magnet, aspirate out supernatant and repeat buffer washing. Aspirate out supernatant completely. For QSC kit, you will need to add 500 μl and centrifuge at 4ºC 2500 rpm for 5 min. Remove supernatant and repeat.
14. **IN DARK.** Add 250-400 μl of FACS buffer (depending on how dilute you want your bead solution) to tubes. Cover FACS tubes in Styrofoam racks with paraffin and foil and proceed to FACS. Place racks in large foam box for convenience. Remember to take thumb drive, notebook with FACS settings, key and access card and of course samples.

**NOTE: All staining expts were performed with 1 μl of beads in 10 μl coating volumes to avoid error. Antibody and DLL4 amounts were scaled down accordingly.

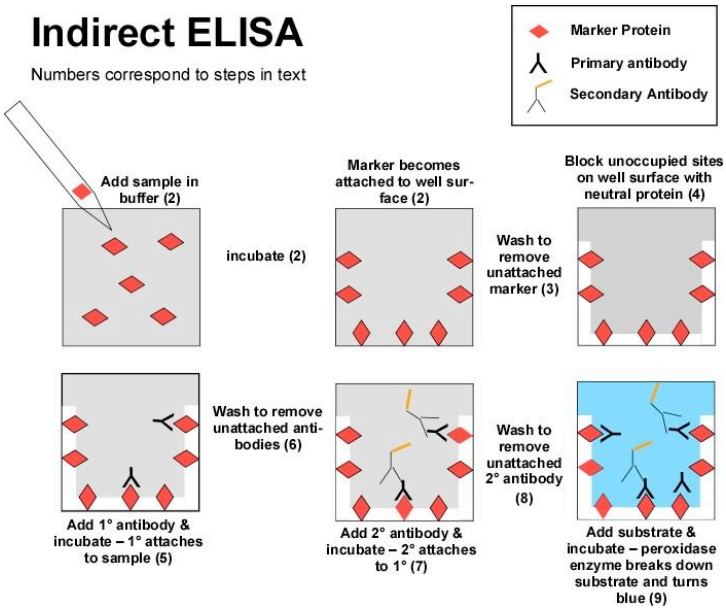
Created by: Sabia Taqvi
Revised: 4/23/06

Indirect DLL4 quantification using ELISA analysis

MATERIALS

96 well high protein binding ELISA plate (Corning, NY)
DLL4 (R&D Systems, 4°C)
Anti-DLL4 antibody (R&D Systems, -20°C)
HRP Goat anti-rat antibody (eBiosciences, -20°C)
Washes from DLL4 staining (after DLL4 incubation, save 0.5-1ml washes with 0.1% BSA in PBS in low protein binding tubes at -20°C)
Coating buffer (0.1% BSA in PBS, prepare fresh)
Washing buffer (0.5% (v/v) Tween 20 in PBS (PBT))
Blocking buffer (1% (w/v) bovine serum albumin in PBS)
Tetramethylbenzidin ((TMB), R&D systems, 4°C)
Stop solution (0.3M sulfuric acid, RT)
0.1-10 μl, 2-20 μl and 100-1000 μl pipettors with tips
Yellow and blue tips
Opsys MR Microplate reader (Dynex. Technologies Inc., VA)
Revelation QuickLink software (Thermo Labsystems, VA)

CONCEPT:



from "http://entomology.tfrec.wsu.edu/VPJμl ab/images/indirect_elisa.jpg"

METHODS

1. To quantify the amount of DLL4 on beads, we will indirectly measure the DLL4 using the washes from DLL4 fabrication. Basically, while fabricating beads, save all three washes in low protein binding tubes. In addition to washes from n=3 samples, save original DLL4 amounts in 0.1% BSA in PBS in low protein binding tubes. Store tubes in -20°C until ELISA analysis.
2. Add 100 μl of each wash sample along with original DLL4 concentrations to wells of 96 well EIA/RIA ELISA plates and incubate over night at 4°C. Also, add DLL4 standards and 0.1% BSA in PBS (background) to additional wells and incubate as well.
3. Wash plates 4x with 100 μl of 0.5% (v/v) Tween 20 in PBS (PBT).
4. Block wells with 1% (w/v) bovine serum albumin in PBS for 1.5 h at 37°C.
5. Washed 4x with 100 μl of PBT.
6. Add 100μl of rat anti-mouse DLL4 IgG2a at a concentration of 1 μg/ml to each well and incubate for 1.5 h at room temperature (RT).
7. Wash 4x with 100 μl of PBT.
8. Add HRP conjugated goat anti-rat IgG at a dilution of 1:5000 to each well and incubate for 1.5 h at RT.
9. Wash 4x with 100 μl of PBT.
10. Add 100μl of TMB to each well and incubate for 10 – 60 min (30 min should be good enough) at RT.
11. Reaction can be stopped by the addition of 50μl of stop solution to wells.
12. Read absorbance values at 450 nm using the Opsys MR Microplate reader and Revelation QuickLink software. Turn on reader 5 minutes before ending and press cancel 7 times (errors). Once reader ready, only then open software or error will occur. Select ELISA HRP program from list of tests and read.
13. Produce standard curve and subtract the measured amount of DLL4 in the washes from the original amount of DLL4 added to the beads to find amount of DLL4 adsorbed onto DLL4 beads.

Created by: Sabia Taqvi
Revised: 5/5/07

MTT Viability Protocol

MATERIALS

MTT (5mg/ml in Hank's balanced salt solution (Invitrogen) – SigmaAldrich) Store at -20°C, stable for 6 months at -20°C
MTT solubilization solution - 10% Triton X-100, 0.1 N HCl in anhydrous isopropanol - Store at 4°C
1.5ml centrifuge tubes with pointed ends
Microcentrifuge
Plate reader with filter at 550nm
Relevant medium (prewarmed)
96 well ELISA plates
ASSUMPTION: preplated cells in 96 well plates
Sterile blue and yellow pipette tips
20-200μl pipettor
2-20μl pipettor

METHODS

1. Remove preplated cells in 96 well plate and remove medium from each. Use separate pipette tip for each group.
2. Add 100μl of fresh prewarmed medium to each well. Do NOT forget to have wells with only medium that you use as background.
3. Add 10μl (or 10% of medium volume) of MTT solution to each well and mix well.
4. Incubate for 2-4 hours at 37°C (we usually do 4 hour incubations).
5. Add MTT solubilization solution in a volume equal to medium volume (100μl) to each well and pipette several times to dissolve purple formazan crystals.
6. After pipetting, remove supernatant and pipette into prelabeled microcentrifuge tubes.
7. Spin tubes for 5 min at 300 rpm to precipitate any cell/debris.
8. Remove 100μl from each tube and place into 96 well ELISA plate.
9. Spectrophotometrically read absorbances at a wavelength of 570nm (or for us 550nm). Shaking for 5 min is not required but is done.
10. Export absorbance values to excel and plot.

Created by: Sabia Taqvi
Revised: July 20, 2007

DLL4 bioactivity assay with C2C12 cells and immobilized and soluble DLL4

MATERIALS

DLL4 (R&D Systems, -20ºC)
Biotinylated anti-6x HIS antibody (R&D Systems, -20ºC)
Neutravidin (Pierce Biotechnology, 4ºC)
DLL4 coated beads and uncoated beads (for bead studies)
96 well tissue culture treated plates
C2C12 myoblasts

Maintenance medium

DMEM (Invitrogen, 4ºC), 10% FBS (Hyclone, -20ºC), pen/strep (Invitrogen, 4ºC)

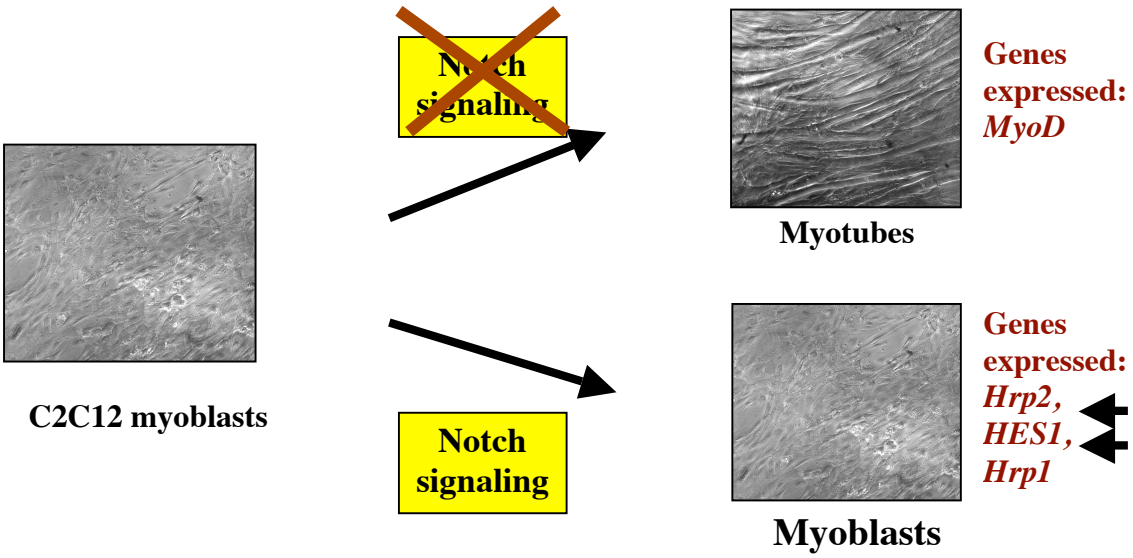
Serum-free differentiation medium

1:1 ratio of DMEM and F12 nutrient mixture (Invitrogen, 4ºC), 1mM sodium pyruvate (Invitrogen, 4ºC), 2.5 μg/ml bovine insulin (Sigma-Aldrich, -20ºC), and antibiotics.

**** Conventional C2C12 differentiation medium**

DMEM (Invitrogen, 4ºC), 10% Horse serum (Hyclone, -20ºC), pen/strep (Invitrogen, 4ºC)
Sterile 50 ml centrifuge tubes
Trypsin (Invitrogen, -20ºC)
Sterile PBS
5-10ml sterile stripettes
Pipettors (2-20, 20-200, 1000)
Sterile yellow and blue tips
70% ethanol
Hemocytometer
Camera and microscope
Thumb drive

CONCEPT:
Immobilized DLL4 inhibits C2C12 myoblast differentiation through Notch signaling. Differentiation is visible through the formation of myotubes or fused myoblasts (size is much larger). With immobilized DLL4, myoblasts maintain their morphology while control wells will show myotube formation within 5-6 days of incubation.



METHODS

1. Maintain C2C12 cells 1 week prior to experiment in maintenance medium.
2. *Day 1:* Check under microscope before use to ensure cells are not overconfluent and there are no traces of contamination.
3. Place ethanol sprayed pipettors, stripettes, tubes, and plates under UV light in hood at least 20 minutes prior to expt beginning.
4. Thaw out DLL4 from -20ºC before use. For previous studies, 1 μg/ml of immobilized DLL4 has been sufficient to inhibit C2C12 differentiation.
5. Coat 96 wells with 1 μg/ml of DLL4 in sterile PBS in 100 μl volume. Perform study in at least n=3. Incubate at 37ºC for 2-3 hours.
6. Thirty min prior to incubation end, begin cell culture. We will need at least 20000 cells/well, thus one confluent 25cm^2 flask will suffice. Ethanol maintenance medium, trypsin and PBS well and bring into hood.

7. Aspirate out old medium and wash with trypsin. Add fresh trypsin and incubate for 10-15 min at 37ºC.
8. Remove flask and check under microscope to make sure cells have detached from flask.
9. Wash flask with 5 ml of fresh maintenance medium and transfer trypsinized cells + medium into centrifuge tube. Pipette well to remove aggregates.
10. Pipette out 10 μl of cell suspension and add to hemocytometer. Count and record cell count.
11. Cap centrifuge tube and spin down at 300 g for 5 min.
12. After centrifugation, remove tube, ethanol tube and bring into hood. Calculate amount of maintenance medium necessary to add to achieve 20000 cells/.1 ml concentration. Remove supernatant and resuspend cells in calculated medium. Set aside tube.
13. Remove plate from incubator and bring into hood. Remove DLL4/PBS and wash wells with 100 μl of PBS 3x.
14. Add 100 μl of cell suspension to wells (cells only, immob DLL4, soluble DLL4).
15. Add 0.1 μg of DLL4 to soluble wells.
16. Adjust all wells to a volume of 200 μl of maintenance medium. Place plate into incubator.
17. *Day 2:* After one day of incubation, remove medium. Add fresh differentiation medium to wells (remembering to add soluble DLL4 to appropriate well).
18. Check cells daily for contamination and differentiation progress.
19. *Day 5/6:* Cells be ready for imaging. You should see definite myotube formation in cells only and soluble DLL4 conditions and only myoblasts with immobilized cells. Image wells with microscope and save on thumb drive. After imaging, dispense plate into trash.
20. *For bead studies,* follow the protocol described above. Seed cells one day prior to bead addition. Prepare bead-differentiation medium suspensions in individual tubes (with extra medium for _-1 well), mix well, and add on Day 2 once old medium is removed. Continue with protocol described above.

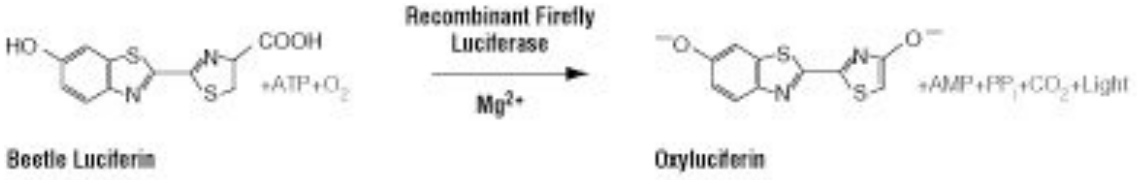
Created by: Sabia Taqvi
Revised: 6/25/07

Transfection and Luciferase Protocol using Exgen 500 and Glo Lysis Buffer

MATERIALS

24 well plates
C2C12 cells
Trypsin
C2 maintenance medium (DMEM, 10% FBS, P/S)
C2 serum-free differentiation medium (1:1 DMEM/Ham's Nutrient Mixture (Gibco), 2mM l-glutamine, P/S, 1mM sodium pyruvate, 2.5 ug/ml bovine insulin (Sigma))
DMEM
FBS
P/S
DLL4 coated beads, uncoated beads
DLL4
4xwtCBF1Luc plasmid (1.8 mg/ml) (Gift from D Hayward, amplified by Aldevron)
Exgen 500 (Fermentas)
Glo lysis buffer (Promega)
Black luminometer plates
BCA assay reagents (Pierce)
96 well plate
PBS
Bovine serum albumin
Pipettors
Yellow and blue tips
Dynalbiotech magnet
Sterile tubes for storing lysates
Plate reader
Luminometer (MBB key)
Luciferin (-80°C)
NaCl
ddH2O
Filter syringe
Filter (0.2 μm)
Vortex
Insulin syringes
Sterile 15 ml centrifuge tubes

CONCEPT:



From Promega notes:

"Firefly luciferase is by far the most commonly used bioluminescent reporter. This monomeric enzyme of 61kDa catalyzes a two-step oxidation reaction to yield light, usually in the green to yellow region, typically 550–570nm (Figure 8.1). The first step is activation of the luciferyl carboxylate by ATP to yield a reactive mixed anhydride. In the second step, this activated intermediate reacts with oxygen to create a transient dioxetane that breaks down to the oxidized products, oxyluciferin and CO_2. Upon mixing with substrates, firefly luciferase produces an initial burst of light that decays over about 15 seconds to a low level of sustained luminescence."

METHODS

1. <u>*Cell seeding*</u>. Maintain C2C12 cells in C2 maintenance medium. Trypsanize and seed cells into 24 well plate 24 hours prior to transfection time so that cells will be 60-80% confluent at the time of transfection. Higher confluencies will result in reduced transfection efficiency. Seeding 40-60000 cells per well usually suffices. If possible, seed extra well for counting.

   NOTE: Each condition will have an n=3 for the control plasmid. If testing concentration of beads, make sure you allot for protein coated and uncoated beads, as well.

2. <u>*Transfection.*</u> We will be using Exgen 500 and the manufacturer's instructions for transfection of reporter constructs. DNA-exgen 500 complexes need to be made fresh prior to transfection.

   a. <u>DNA/Exgen complex formation.</u> First, prepare 150mM NaCl buffer in ddH2O. You will need 100 μl per sample. If you have 96 wells for example, you will need at least 10 ml. For our purposes, prepare 15ml of NaCl. (150mM NaCl x 15 ml = 2.25 mmoles; 2.25 mmoles x 58.4428 g/mole = 0.1315 g => 131.5 mg of NaCl + 15 ml of ddH2O.)

   b. **In Hood.** Label 15ml centrifuge tubes with plasmid and control. Place pipettors, syringes and tubes (ethanoled) under UV light in hood 20 min

prior to complex formation. You want to add your DNA (plasmid) at 1 μg DNA/100 μl of 150mM NaCl buffer. Since we have 5 ml (total) for example per plasmid, we will need 50 ug of DNA. Add plasmid and control plasmid to 4.835 ml of buffer in labeled tubes. Vortex gently (on minimum speed) and spin down.

c. **In Hood**. Pipette out 3.3μl of Exgen x 50 = 165 μl of Exgen and place into sterile centrifuge tube. Fill insulin syringe with Exgen from tube. Holding 15 ml plasmid tube buffer on minimum speed on vortex, add exgen drop by drop into tube until all volume has been added. Incubate for 10 min at room temp.

d. Complex addition. **Cell culture is all performed in hood.** Remove cell culture plates from incubator. Aspirate old medium. Add 400 μl of prewarmed DMEM + P/S. Next, add 100 μl of Exgen/DNA mixture to each well. Rock plate back and forth and from side to side to achieve even distribution. Incubate for 4 hours at 37C.

e. After 4 hours, add 500 μl of DMEM + 20% FBS + P/S. Incubate for another 20 hours at 37ºC.

3. *Bead addition*.

a. Place pipettors, magnet, tubes, and tips under UV light 20 minutes prior to bead addition. Check cells under microscope to make sure NO contamination and viability.

b. Remove old medium from well designated for counting, wash with trypsin, and add fresh trypsin. Incubate for 5 min and wash with medium. Count using hemocytometer and record cell number. You will need this for bead calculations.

c. With cell number, make calculations of how much of bead volume you will need for each bead to cell ratio. For example, if the cell count is 100000 cells/well and you have an n=3 for DLL4 coated beads, you will need at least 350000 beads (have some extra). The beads are stored at 10^7 beads/25 μl. This means you will need .875 μl of beads. Also, you need 1ml of serum-free differentiation medium per well. Since we're doing a little extra, you will need 3.5 ml.

d. Wash necessary amount (1 μl) coated and uncoated beads with 0.1% BSA in PBS (4ºC) with 500 μl and resuspend in original volume but with differentiation medium.

e. Add beads to tubes with medium and mix well.

f. Remove old medium from cell culture plates. Add bead-medium suspension to each well.

g. Check under microscope to make sure beads well suspended. Do not forget to add medium to immobilized DLL4 condition and cells only condition.

h. Incubate for 24 hours at 37ºC with 5% CO2.

4. *Lysis, BCA assay, and luminescence detection.* We will be using the Glo lysis buffer to lyse cells and expose luciferase produced. It is important to lyse cells well (bead-cells tend to aggregate) and avoid bubbles (reduce volume). For normalization of cell number, we perform a BCA assay where a portion of the lysate is tested for protein content. The luciferase readings from the luminescence are then divided by the protein content to get RLU/mg. Thus, it is important to have enough for luminescence and BCA. We usually use 150 μl Glo lysis buffer.

a. Lysis: Check cells for contamination, cell death and note any differences between wells.

b. Place Glo Lysis buffer (4ºC) in lukewarm water bath to equilibrate to room temp 20 min prior to lysis. Warm PBS in water bath (37ºC).

c. Remove old medium from cell culture wells (be sure to use different tips for different conditions). Wash with 500 μl of PBS and remove.

d. Add at least 150 μl of Glo Lysis buffer to each well. Incubation of 15 min at room temp is desired but to achieve complete lysis, a longer incubation time is recommended. After 15 min of incubation, pipette wells to lyse cells. After an additional 20-30 min, pipette several times to achieve a homogeneous lysate (no clumps or aggregates). Place these plates on ice or at 4ºC while preparing BCA assay.

e. **BCA:** Prepare BSA standard solutions (stock (2 mg/ml) from Pierce BCA kit) in PBS. Concentrations should be made in lo protein binding tubes using serial dilutions and include 0 μg/ml, 2.5 μg/ml, 10 μg/ml, 20 μg/ml, 40 μg/ml, 200 μg/ml and 400 μg/ml. Add 50 μl to each well in 96 well plate (ELISA plate is fine). Do a n=3.

f. Prepare the BCA reagent. The ratio is 25:24:1 for Reagent A:Reagent B:Reagent C, respectively. Prepare enough for standards and samples with little extra. Mix well. Prepare plate-plan in lab notebook for both BCA assay and luciferase assay plates.

g. Add 50 μl of BCA reagent to standards and wells designated for samples. Add 50 μl of well-pipetted sample lysate to wells on ELISA plate. Cover plate and incubate for at least 2 hrs at 37ºC.

h. Luminometer: Add 50 μl of well-pipetted samples to black luminometer plates.

i. Remove luciferin from -**80**ºC and let equilibrate to room temp for 15-20 min.

j. Take black plate, luciferin, thumb drive to MBB (Klaus area).

Illuminometer

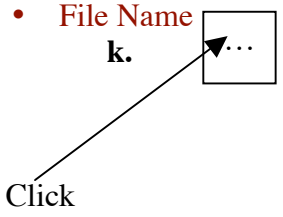
- **Grab keys/access cards**
- **At ICMB make entry into the Log book and switch the instrument ON**
- **Chose software Mikronim 2000.**
- **OPEN→DESKTOP → Bilal (*par files)**
- **Options →measurements ⊙ → select area → inj 3⊙ → Make area in circles red by selecting them**

- Instrument → wash → let it run → inj 3
- Wash again
- Instrument → Prime → inj 3

(Make sure you deselect all else)

- Put plate → Read
- File Name → c: → users → Bilal → Start

k.



BCA: After 2 hours, remove plate from incubator and run assay for BCA assay on plate reader. Record results and print out for lab notebook.

Created by: Sabia Taqvi
Revised: January 4, 2007

First-strand cDNA synthesis protocol using Superscript III (Invitrogen)

MATERIALS

Random hexamers
10mM dNTP mix
DEPC treated water
10X RT buffer
25mM $MgCl_2$
0.1 M DTT
*RNAseOUT recombinant ribonuclease inhibitor **
*Superscript III RT**
*RNase H **
RNA samples (stored at -20°C)
Nuclease-free 1.5ml centrifuge tubes (autoclaved)
Nuclease-free PCR tubes (autoclaved)
RNAse zap
Kimwipes
20% bleach solution
Ice and ice bucket
Float for water bath for PCR tubes
0.1-10 μl, 20-200, 1000 pipettors
water bath (at 65ºC), thermocycler
Lab marker
Foam box for transfer
MBB card and keys

***All italicized reagents are included in the Superscript III First-strand cDNA synthesis kit (catalog no:* 18080-051)

* Starred items are given in limited quantities and will most likely run out. Make sure you have enough for your studies.

METHODS

The 20 _l total volume reaction can be used for 1 pg-5 μg total RNA or 1pg - 500 ng poly(A)*RNA.

1. <u>*Before you start.*</u> Reserve time on thermocycler (coreweb.icmb.utexas.edu) for 2 hours. It'll take you 1 hour or less to prepare the following. Allow for this time accordingly. Spray pipettors with 20% bleach and leave pipettors to dry 10 min before use. Take out kit components (make sure you have enough of each) and leave outside to thaw. Set water bath to 65ºC and add sign saying NOT at 37ºC.

a. Spray ice bucket with 20% bleach and fill with crushed ice. Spray outside with 20% bleach and bring to work area.
b. Once components have thawed, briefly centrifuge all components. Spray with 20% bleach and bring into work area. Keep on ice.
c. Remove DNased samples and spray with bleach and bring into work area. Keep on ice.
d. Label all PCR tubes for samples. Each sample will have a corresponding wRT and a noRT tube. Using the numbers below, calculate how much of each component you will need and make stock solutions. For example, you will need a "Step 1," "with RT," and "without RT." The Step 1 will be for all samples during the denature step. Also, when calculating, allow for error. For example, if you have 20 samples, make for 25 samples.

2. Add the following to each sample:
   - ***1*** *µl* ***l*** *of random hexamers*
   - ***10pg-5ug*** *total RNA (cannot exceed 8 µl l volume)*
   - ***1*** *µl* ***l*** *of 10 mM dNTP mix*
   - *nuclease free water to* ***10*** *µl* ***l***

3. Heat mixture to 65ºC for 5 minutes in water bath using float.

4. Incubate on ice for at least 1 minute.

5. Add the following to each **<u>with RT</u>** sample:
   - ***2*** *µl 10X RT buffer*
   - ***4*** *µl 25 mM MgCl2*
   - ***2*** *µl 0.1 M DTT*
   - ***1*** *µl RNaseOUT recombinant RNase inhibitor*
   - ***1*** *µl of Superscript III RT*

6. Add the following to each **<u>without RT</u>** sample:
   - ***2*** *µl 10X RT buffer*
   - ***4*** *µl 25 mM MgCl2*
   - ***2*** *µl 0.1 M DTT*
   - ***1*** *µl RNaseOUT recombinant RNase inhibitor*
   - ***1*** *µl of nuclease-free water*

7. Mix by pipetting gently up and down. Place tubes in foam box and proceed to MBB.

8. *<u>Thermocycler.</u>* Switch on (switch on right side back). Go to Edit and select any program. Using keys, insert the following program:

- Incubate at 25ºC for 10 minutes and then 50°C for 50 minutes
- Terminate reaction at 85ºC for 5 minutes and then chill on ice (4ºC for 99:59 (inf))

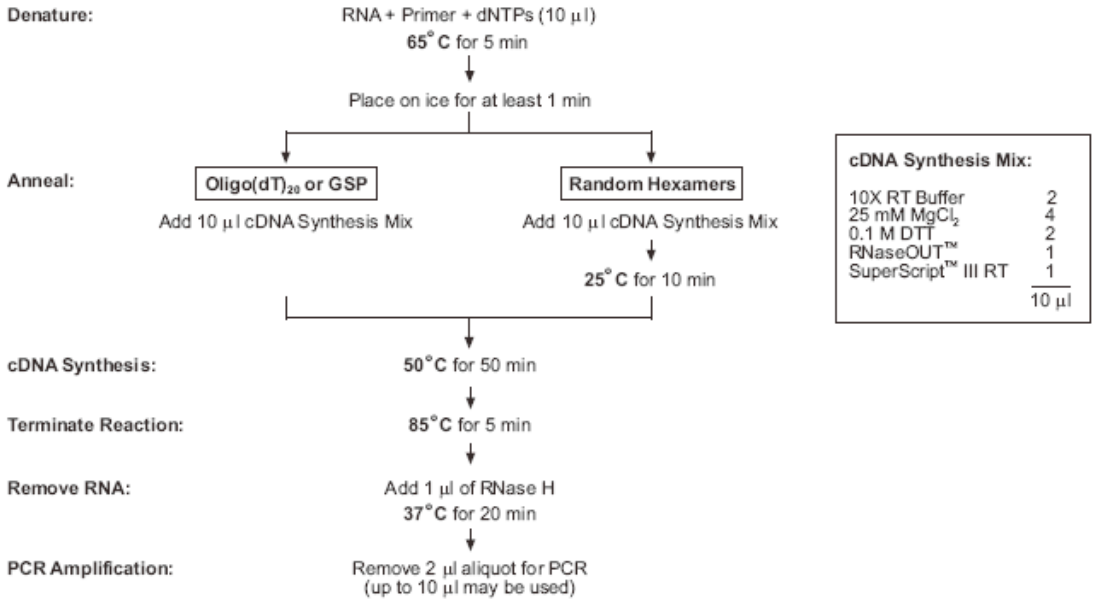
The volume is 20 μl. This will most likely take 2 hours or so. Reset water bath to 37°C.

9. After 2 hours, briefly centrifuge contents.

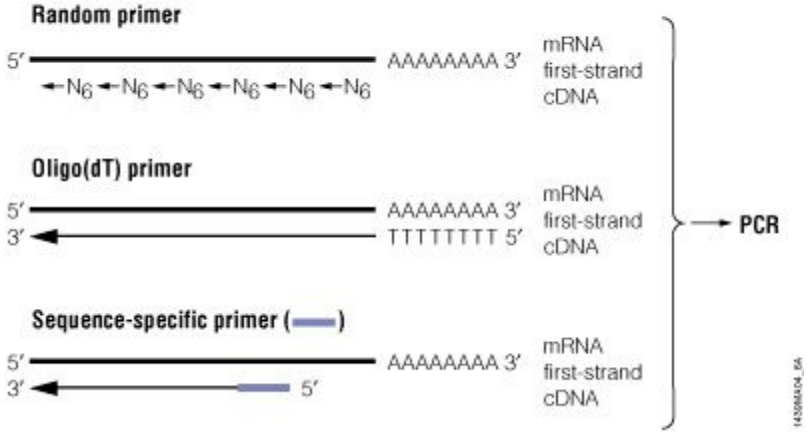
10. Add ***1*** μl *of RNase H* and incubate at 37°C for 20 minutes (to remove complementary RNA).

11. Store cDNA reaction at -20°C or use for PCR immediately.

CONCEPT:



First Strand Synthesis:



Created by: Sabia Taqvi
Revised: July 25, 2007

GORE RNA Extraction Protocol

Reference: Deena Walker, Kimberly Hillsman (Gore Lab)

MATERIALS
Cushion buffer (0.4 ml/sample)*
Lysis buffer (0.5 ml/sample)**
10X SET buffer (70 μl /sample)***
5 M NaCl (21μl /sample)
Proteinase K (Roche, -20C, 14 μl /sample)
1:1 Phenol:chloroform solution (600 μl /sample) – don't forget to remove aqueous layer
Chloroform (600 μl /sample) – prepare fresh in 50 ml centrifuge tube
100% Isopropanol (900 μl /sample)
70% Ethanol in nuclease-free water (1 ml/sample)
RNase-DNase free 1.5ml centrifuge tubes
50ml DNase free-RNase free centrifuge tubes (Corning)
Microcentrifuge tube racks (at least 5-6)
Kimwipes (large)
RNase-zap
2-20 μl pipettors, 20-200 μl pipettors, 100-1000 μl pipettors
ART barrier pipette tips (0.1-10, 20-200, 100-1000 **2 boxes should suffice**), National barrier tips (0.1-100)
Ice bucket with crushed ice
Centrifuge with small rotor at 4°C

Note: ALL solutions (except for phenol:chloroform and chloroform which should be stored at room temperature until use) should be kept on ice. Solutions should be prepared fresh and used within 1 week. All buffers should be made with nuclease-free water.

Note again: Remove RNAse from pipettors, centrifuge tubes, racks, crushed ice in ice bucket and tips by spraying with RNase-zap and placing all in hood under UV for 30-40 minutes prior to use. Adjust microcentrifuge temperature to 4°C.

Note: When weighing chemicals, do not dump extra chemicals back into original container. If using a couple of days old buffers, check pH prior to using.

***Cushion buffer recipe:**
1. Prepare in sterile 50 ml centrifuge tube.
2. Weigh out 5.5 g of sucrose and dissolve in 35 ml of nuclease-free water.
3. Add 400 μl of 1M Tris-HCl (pH 7.4).

4. Add 60 μl of 1M MgCl2.
5. Bring up to 40 ml final volume.
6. Mix and store at 4ºC for up to 1 week.

****Lysis buffer recipe:**

1. Prepare in sterile 50 ml centrifuge tube.
2. Weigh out 4.1 g of sucrose and dissolve in 35ml of nuclease-fee water.
3. Add 100 mg of Na Deoxycholate (Dissolve completely)
4. Add 400 μl of 1M Tris-HCl (pH 7.4)
5. Add 60 μl of 1M MgCl2
6. Add 200 μl of NP-40 (Nonidet P-40, Roche, 4C) *** very viscous
7. Mix and store at 4ºC for up to 1 week.

*****SET buffer recipe:**

In a 50ml centrifuge tube add the DEPC water to the other components to create a total volume of 10 ml.

- 7 ml DEPC treated water
- 1 ml of 1M Tris-HCl (pH 8.0)
- 1 ml of 0.5M EDTA (pH 8.0) heated at 56˚C to solubilize
- 1 gram SDS

Mix and store at 4˚C or on ice for up to 1 week.

Phenol:chloroform:isoamyl alcohol (25:24:1):

Prepare this solution fresh on the day of RNA isolation. You will need 600 μl of this per sample, calculate total amount need and make solution accordingly. When preparing, use glass pipettes and avoid aqueous layer in phenol. Remove aqueous layer after adding both chloroform and phenol to tube using 1000 μl pipettor.

METHODS

Day 1

1. Label 4 RNase-free/DNase-free tubes (marked 1a, 1b, 1c, 1d for example) per sample and place in centrifuge tube racks.
2. Add 400 μl of cold cushion buffer to each "a" tube of set.
3. Add 500 μl of cold lysis buffer to frozen samples and pipette up and down several times to lyse cells completely. Layer this over the cushion buffer so fractions do not mix.
4. Centrifuge "a" tubes in microcentrifuge at 800 g for 6 minutes at 4ºC. Return samples to ice bucket after spin.
5. Transfer upper phase (550 μl) of tubes to tubes marked __b.
6. In these tubes, add 70 μl of 10X SET buffer, 21 μl of 5M NaCl, 14 μl of PK (10mg/ml). Vortex well (for 15 sec/tube). Vortexing is crucial to making sure the protein is degraded.

7. Incubate for 45-60 at 45ºC. Adjust microcentrifuge temperature to 21°C.
8. After incubation, add 600 μl of phenolchloroform to each tube. Vortex hard and mix well.
9. Centrifuge tubes at 19000g at 21°C for 4 minutes.
10. Transfer upper phase to tubes marked __c. Add 600 μl of chloroform to tubes. Vortex hard and mix well.
11. Centrifuge tubes at 19000g at 21°C for 4 minutes.
12. Transfer upper phase to tubes marked __d. Do not get too close to protein layer or organic layer. This will lower quality of RNA.
13. Add 900 μl of ice cold 100% isopropanol, vortex well and store at -20°C for at least 1 hr.

Day 2 Precipitation

MATERIALS
70% Ethanol in nuclease-free water (1 ml/sample, 4°C)
Nuclease-free water (9 μl /sample, 4°C)
Kimwipes
RNase zap
0.1-10 μl pipettor, 100-1000 μl pipettor
Microcentrifuge at 4˚C, fast temp this prior to using
RNA samples
Nanodrop

Note: Remove RNAse from pipettors, centrifuge tubes, racks, kimwipes, crushed ice in ice bucket and tips by spraying with RNase-zap and placing all in hood under UV for 30-40 minutes prior to use. Adjust microcentrifuge temperature to 4°C.

METHODS
1. Centrifuge the RNA tubes at 4˚C at 19,000g for 20 minutes. Make sure all the tubes are place with the tabs pointing out. This makes it easier to locate each pellet.
2. Carefully remove the RNA tubes trying not to disturb the pellet.
3. Carefully pour off the isopropanol making sure not to pour off the pellet. Use a sheet of colored paper if necessary to make it easier visualize the pellet.
4. Add 1ml ice cold (-20˚C) 70% EtOH (made with nuclease free water) to each sample. Cap and invert the tube a few times to wash the pellet.
5. Centrifuge at 4˚C at 19,000g for 10 minutes.
6. Again carefully pour off the EtOH making sure not to pour off the pellet. Remove as much EtOH as possible. Leave tubes open and inverted over a kim wipe. Allow the EtOH to evaporate for 1 hour.

7. If after 1 hour, there is still a significant amount of EtOH not evaporated, used a kim wipe to soak up some EtOH or pipette off small amounts to leave as little EtOH as possible.
8. Resuspend the pellets in 9 μl of nuclease-free water.
9. Vortex and pulse in the room temperature microcentrifuge to concentrate the contents at the bottom of the tube.
10. Proceed to MBB for nanodrop measurement. Take 0.1-10 μl pipettor and 0.1-10 μl RNase free tips with samples and place in foam box. Also, bring notebook to note down absorbance values. You will need biosci username and password. Measure with the Nanodrop (MBB)and store at -80˚C. Absorbance values should be greater than 1.8 for both 230:260 and 280:260 ratios.

Created by: Sabia Taqvi
Revised: 3-1-07

Turbo DNase kit protocol (Ambion)

MATERIALS:
TURBO DNase
10X TURBO DNase buffer
DNase Inactivation reagent
Nuclease free H2O
Ice
Ice bucket
Water bath at 37C
0.1-10μl pipettor
Centrifuge
Timer
Kimwipes
10% bleach solution
RNA
0.5ml tubes

NOTE: This may be the MOST crucial kit in preventing curves in your negative control for real time RTPCR. It is important to aliquot your DNase into low protein binding tubes and do not expose these to repeat freeze-thaw cycles. It is also important to mix tubes well during incubation and inactivation. All of this will ensure proper DNase treatment.

METHODS:

1. Add 0.1 volume 10X TURBO DNase buffer (1 μl) and 1_l TURBO DNase to RNA (this can remove up to 2 ug of DNA) into 0.5ml tubes.
2. Incubate at 37C for 20-30 minutes.
3. Add resuspended DNase Inactivation reagent (0.1 volume = 1.1μl). Mix well.
4. Incubate for 2 min at RT, mixing occasionally.
5. Centrifuge at 10000g for 1.5 minutes and transfer to fresh tube. This volume is usually near 7-7.5 μl. **DO NOT aspirate inactivation reagent,** may interfere with enzymatic reactions.

Created by: Sabia Taqvi
Revised: Feb. 2, 2007

DNA Gel Electrophoresis

Materials

Comb (10 vs. 20 wells)
Agarose
1L of TBE buffer**
Gel casting tray, chamber & electrodes, voltage generator
Loading buffer
DNA ladder
DNA samples
Parafilm
2-20 μl pipettor
0.1-10 μl pipettor
0.1-10 nuclease-free tips
2-20 nuclease-free gel loading tips
200-300ml of DDH2O
Staining container
Orbital shaker
UV transilluminator
Weigh boats
Graduated cylinder
Spatula
Ethidium bromide

Methods

1. *TBE buffer preparation.* First step is to make TBE buffer for running gel.

****1x Tris-Boric Acid-EDTA Buffer (TBE) Recipe** – 89mM tris (pH 7.6), 89 mM boric acid, 2 mM EDTA
To make 10x stock (1 liter) – dissolve the following in 600ml of distilled water and then fill to final volume of 1 L with distilled water:

108 g Tris base (FW: 121)
55 g Boric acid (FW: 61.8)
40 ml 0.5 M EDTA (pH 8.0)

2. *Gel preparation.* Since the genes we're looking at are near 100 bp, we will prepare a 2 (w/v) % agarose gel. Consult the following chart for other gene sizes and appropriate gel concentrations.

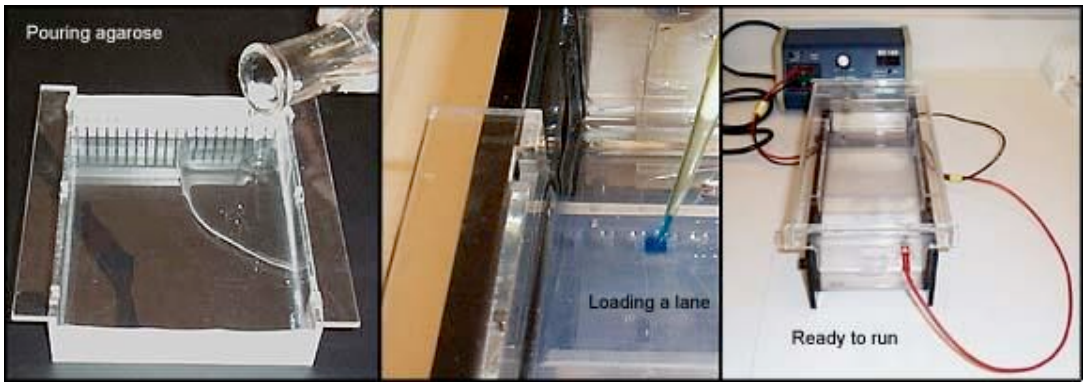
Gel Concentration (%)	DNA Size (Kb)
0.50	1–30
0.75	0.8–12
1.00	0.5–10
1.25	0.4–7
1.50	0.2–3
2–5*	0.01–0.5

* Sieving agarose such as AmpliSize® agarose

a. Weigh out 2g of agarose into a weigh boat and measure 100ml of TBE buffer in a graduated cylinder.
b. Add buffer into a small flask (able to fit into microwave) and add agarose into the liquid. Swirl around several times to mix.
c. Place flask into microwave and microwave on high for 2 min. Be careful NOT to let mixture bubble over otherwise you will have a mess.
d. Also, add appropriate comb (based on how many samples you have) into tray prior to heating up mixture. Tape edges well to prevent agarose from spilling over.
e. You will need to heat up the flask several times to make sure agarose has dissolved (turn off lights to aid in watching). Once dissolved fully, pour 80 ml of mixture into tray and using spatula, remove any debris, undissolved fragments (turn on the light to aid in seeing). Leave tray undisturbed for 20-30 minutes.

3. *Sample preparation, loading and running gel.*
   a. While gel is cooling, prepare samples. You will need a long sheet of parafilm to mix the samples on. Paper does not need to be removed.
   b. Prepare chart in lab notebook with information on what sample goes into which well.
   c. When preparing samples, prepare them in the order to be loaded. First well is usually DNA ladder.
   d. For our tray, add 5-6μl of DNA loading buffer and 15-20μl of DNA sample (only 10-12μl of DNA ladder should be appropriate) using appropriate tips.
   e. Once gel is cooled, remove tape and comb, carefully.
   f. Place tray with gel in the appropriate position (wells at the top) into chamber. Add TBE buffer until "fill to this line." (about 700-800ml). Do not pour on gel.
   g. Once chamber and gel are ready, mix each sample by pipetting and load into gel using loading tips. Position tip right above well so that sample goes to desired well.

h. Once samples are loaded, place lid on gel tightly, connect electrodes to power supply (making sure samples are running from negative to positive) and turn on supply. Increase voltage to 110 V and press run.
i. Check on gel from time to time to make sure you see bands of loading buffer moving.



4. <u>*Staining gel.*</u> Once bands have run 50-60% of gel length, then power supply can be turned off.
    a. Remove lid from chamber and remove casting tray with gel, being careful not to let gel slip out.
    b. Using graduated cylinder, measure 200-300ml of TBE buffer from chamber by pouring from chamber to cylinder in sink. Add liquid to staining container.
    c. We will need 0.5 μg/ml Ethidium bromide solution to stain the gels. Thus, you will need 100-150 μg of Et br. Add this to solution and mix well before adding gel.
    d. Slip in gel carefully to container to prevent splashing. Close and place on orbital shaker for 15-30 min.
    e. Remove liquid from container and add 200-300ml of ddH20 into container, making sure not to add it directly onto gel. Close container. Destain on orbital shaker for 10-30 min.
    f. Remove liquid and rinse gel with ddH2O briefly. Add fresh ddH2O to cover gel, close container, place container with several pairs of gloves and napkins in Et br box and proceed to MBB for imaging. (Don't forget access card if after hours.)

5. *Imaging gel.* Imaging rooms are on 2nd and 3rd floor. If they are in use, they will be locked. If vacant, they will be kept open with a wedge. When finished using, place wedge between door and wall to keep open.
    a. Wear gloves, turn on regular light from illuminator and turn off light of room.
    b. Click on alpha imager (software), type in ROBERT and KRUG for username and password.
    c. Remove gel from tray carefully and place on illuminator.
    d. Increase the exposing time and adjust the black, white and gamma channels to reduce background and see bands most clearly. Use the camera lens for focus and zooming in.
    e. Once image is ready, select freeze and print image. Tear off image and keep for records.
    f. Close program, remove gel from illuminator and place into container. Wipe illuminator with napkins, remove gloves and return to lab.
    g. Dump water in sink, wash container and throw gel into biowaste.

Created by: Sabia Taqvi
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Negative control Real-time RT-PCR using SYBR green/ROX Superarray Mastermix

Real-time must be performed on all samples without RT to confirm the absence of DNA contamination in samples (DNase treatment should have removed all DNA, but process is a good check to see if samples can be run using real-time). Also, must perform no template reaction to make sure no background, confirm the adequate linear range.

MATERIALS:
SYBR green/ROX RT^2 Real-time PCR master mix (Superarray, cat no: PA-112)
dd H_2O
undiluted/diluted template
primer set
real-time optically translucent 384 well plate (can be obtained from core facility, see Shawn)
clear tape (see Shawn)

METHODS:

1. For each 25ul reaction, mix the following in an optically clear 384 well plate:
   - 12.5 μl RT^2 Real-time PCR master mix
   - 10.5 μl dd H_2O
   - 1 μl template without RT
   - 1 μl RT^2 PCR primer set

2. For the no template reaction negative control, mix the following in an optically clear 384 well plate:
   - 12.5 μl RT^2 Real-time PCR master mix
   - 11.5 μl dd H_2O
   - 1 μl RT^2 PCR primer set

3. Take plate to core facility and proceed with real-time methods.

Created by: Sabia Taqvi
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Flow cytometry staining for hematopoietic progenitors protocol

MATERIALS:
FACS tubes
Microcentrifuge tubes
Crushed ice
Foam box
Pipettor (2-20μl, 100-1000μl)
Yellow tips
Blue tips
Ice cold FACS buffer (1% BSA, 0.05% Na Azide in PBS)
White rack for facs tubes
Parafilm
Zip disc/Thumb drive
Antibodies (mouse cKit-FITC, sca-1-PE, CD16/CD32 FcBlock (BD Pharmingen))

PROCEDURE:
Prepare staining scheme prior to experiment, complete with images, how each sample will be stained for what. Check all antibodies prior to staining and consolidate antibodies in box. Prepare calculations for staining protocol (how much antibody, how much FACS buffer). Staining volume is usually 50μl /million cells. Recommended antibody concentrations are listed on antibody datasheets. Common concentration is 1μg/million cells. (samples, extra unstained sample, isotype controls, propidium iodide)

Staining

1. Wash pelleted cells twice with FACS buffer and spin at 300g for 5 minutes, 4 ºC. For second wash, resuspend pellet in 1ml of buffer and transfer to 1.5ml microcentrifuge tube and proceed as before.
2. Following centrifugation, resuspend in 30μl of FACS buffer.
3. Add calculated FcBlock amounts to each sample, resuspend cells, and incubate for 10 minutes on crushed ice.
4. **TURN OFF LIGHTS**. All remaining steps are done in **DARK!!!!**
5. Add calculated amounts of both antibodies to all samples (except isotype control samples), resuspend, and stain for 30 min in dark on ice.
6. For isotype control samples, add isotype control to one sample and stain as above.
7. For extra sample, do not stain.
8. Remove from ice, tap with finger, add .5ml of facs buffer, spin for 5 min.
9. Remove supernatant from samples.

10. Resuspend sample in 300μl of FACS buffer and transfer to FACS tubes in foam racks. For propidium iodide sample, add 10μl of propidium iodide when ready to proceed to FACS and resuspend in FACS tube. Parafilm tubes, cover with foil and place in foam box. Now you are ready to proceed to FACS.

FACS

1. Follow FACS procedure: Turn on pump, turn on facs machine, prime 3x, turn on computer, enter cellquest pro, set up template or open existing template, connect to cytometer, open up threshold, compensation, counters, setup acquisition& storage, open inspector.
2. These next steps are VERY IMPORTANT. Follow strictly:
    a. First run positive control and adjust voltages (FSC, SSC, FL1, FL2). VERY IMPORTANT to adjust compensation, as well. Draw region around positive regions in FL1FL2 plots to backgate in scatter plot and see where lymphocyte populations are located. This will be your reference plot. Record voltages and compensation.
    b. Now, run sample. Make sure speed is low and acquisition number is set to something large. Work fast. Focus on FL1FL2 plot to center cells (negative or positive, doesn't make a difference) in center of plot. Make sure there are no cells not included (pushed down, etc.). If cells are pushed at the top, it is fine, these are most likely antibodies not cells. Adjust compensation to make sure FL1 cells do not look like FL1FL2 positive cells (at the same time, making sure cells are not pushed to the side). Record settings !!
    c. Once settings are set, you can continue acquiring all cells. Settings should not need to be changed. Change label with each sample. Make minor adjustments if necessary.
    d. Follow shut-down procedure. Make a copy of data, stats onto zip, thumb drive. Fill in sign-in sheet.

Created by: Sabia Taqvi
Revised: December 17, 2006

Cell harvesting from scaffold culture protocol

MATERIALS:

Accumax (Innovative Cell Technologies, San Diego, CA)
50ml centrifuge tubes (2 tubes/scaffold)
15ml centrifuge tubes
40um meshes (1 mesh per condition)
Sterile PBS
37°C water bath
Timer
Centrifuge
Cell specific medium
Hemacytometer
2-20μl pipettor

METHODS:

1. Remove scaffolds from 24 well plates and place in 50ml centrifuge tubes using forceps (1 scaffold/tube). Add 1ml of PBS to side of tube.
2. Rinse scaffolds and remove PBS carefully, not disturbing scaffolds.
3. Add 250ul-500ul of Accumax (enough to cover scaffolds) to each tube. Vortex gently and incubate in 37°C water bath for 10 min. (Vortex gently every 3-4 minutes).
4. Detach cells by adding 1ml of serum-containing medium to each tube and gently pipetting repeatedly for 10-20x.
5. Place mesh on top of 50ml centrifuge tube and add detached cell suspension to remove scaffold remnants. Rinse centrifuge tube with 1ml of medium and wash filter to remove and remaining cells.
6. Reuse same mesh for other scaffolds of same condition.
7. Repeat above for each scaffold.
8. Resuspend cell suspension well and transfer to 15ml centrifuge tubes. Count cells from representative sample and note in lab notebook
9. Spin down for 300g at 4°C for 8 minutes and aspirate out supernatant. Pellets should be small but visible.

Created by: Sabia Taqvi
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Scaffold sterilization and seeding protocol

MATERIALS:

70% ethanol
Sterile PBS/ fetal bovine serum/cell specific medium
Centrifuge tubes
Rotator/shaker
Scaffolds
Trypsin
Cell specific medium
Hemacytometer
2-20 μl and 20-200 μl pipettor and sterile yellow tips
60mm sterile plastic plates
60mm circles of parafilm that have been UV lighted prior to cell seeding
24 well tissue culture plates
Sterile forceps
Metal weighing tool
Sterile Pasteur pipette
Centrifuge tube
Pipettes

METHODS:

1. Place scaffolds in individually labeled centrifuge tubes with about 10-20ml of 70% ethanol (enough to cover the scaffolds completely while rotating on side). Then, place tubes on rotator in ENS 621. Rotate for at least 1-2hr.
2. After 1 hr, remove tubes from rotator and bring in hood. Aspirate ethanol and replace with 10-20ml of sterile PBS (or medium/FBS). Rotate on rotator for at least 3 hrs. The PBS wash will remove ethanol from the scaffolds.
3. While scaffolds are washing, trypsinize and count cells. Prepare cell suspension so that cell seeding volume is not more than 50-100μl /scaffold for 6-8mm diameter scaffolds and 150-200μl /scaffold for 10-15mm diameters.
4. Once suspension is ready, ethanol centrifuge tubes with scaffolds and bring inside hood. Using forceps, place parafilm discs in 60mm dishes. Remove PBS wash using glass Pasteur pipette and using forceps transfer sterile scaffolds to paraffin films. Use another Pasteur pipette to remove any residual liquid volume on scaffolds (once you've transferred to parafilm discs). Use different dishes for different scaffold conditions.
5. Mix cell suspension well in centrifuge tube and seed dropwise using appropriate pipettor onto scaffolds carefully making sure volume sits on top of scaffold.

6. Cover dishes, label, ethanol and place in incubator carefully so that scaffolds do not move around. Incubate for 7 hrs.
7. Check scaffolds periodically to make sure volume has not evaporated. If so, add appropriate amount of medium.
8. After 7 hours, prepare 24 well plates by adding 1 ml of fresh medium into individual wells and 1 ml of PBS on perimeter.
9. Transfer scaffolds from dishes to plates, by first immersing scaffolds in medium in centrifuge tubes (to wash off any unattached cells) and then transferring to 24 well plates using sterile forceps. Label, ethanol, and place in incubator.
10. Replace medium every 3-4 days.

Created by: Sabia Taqvi
Revised: 12-12-06

Scaffold fabrication protocol

Adapted from:
Susan L. Ishaug et al. "Bone formation by three-dimensional stromal osteoblast culture in biodegradable polymer scaffolds." Journal of Biomedical Materials Research, Vol. 36, 17–28 (1997)

MATERIALS:
PLLA (Lakeshore Biomaterials)
Organic solvent (methylene chloride/dichloromethane or chloroform)
NaCl
Glass vials
Weigh boats
Sieves for pore sizes (<150μm, 150-425μm, >425μm)
Teflon coated muffin pans
Biopsy punch, metallic punchers (8-10mm diameter)
1-2 L beakers
DDH2O
Spinner plates
Magnetic stirbars
Kimwipes
15 or 50ml centrifuge tubes
Tape or rubber bands

CALCULATIONS:
We use 5ml polymer solution volume / 60m diameter mold. This gives us 2-3mm thickness. For a 10% (w/v), that's 10g/100ml * 5ml = 500mg. For 90% porosity, we use 4.5 g of NaCl.

METHODS:
1. Weigh out 4.5g of NaCl in weighboat (4.5g of salt gives 90% porosity) per mold. Each mold gives 15 8mm diameter scaffolds.
2. Weigh out PLLA according to (w/v) percentage in weigh boat and transfer to glass vials.
3. Add 5 ml of dichloromethane or chloroform to PLLA in vial in chemical hoods using glass pipets and pipet aid, cap on tightly, and vortex till completely dissolved.

4. Add NaCl into Teflon coated pan so that salt layer is evenly dispersed within the mold.
5. Pour polymer solution into mold and shake continuously to achieve even salt dispersion. Using a glass stirrod to evenly distribute salt may help.
6. Let organic solvent evaporate for 1-3hours. Longer periods will ensure complete evaporation but will make scaffold punching more difficult.
7. Invert mold and pop out scaffold disc. Place on kimwipe. Using biopsy punch or metallic punch, punch out scaffolds and let airdry over night. Only punch out if scaffolds fairly dry. Any stickiness/ strings mean you should wait.
8. For salt leaching, fill a 1L beaker with 1L of DDH2O. Place 10-15 pre-cut scaffolds in beaker with appropriate sized stir bar. Turn on stir setting to 3-4 so that scaffolds are moving but not being sheared.
9. Change water every 12 hours and let salt leach out for at least 48 hrs.
10. Remove scaffolds, dry water on kimwipe, and freeze-dry using liquid nitrogen and lyophilizer for at least 48 hrs. To freeze-dry, transfer scaffolds to 15 or 50ml centrifuge tube. Cover with small kimwipe and rubber band or tape. Place in liquid nitrogen for 1 minute and transfer to lyophilizer immediately.
11. After freeze-drying, transfer scaffolds as is to desiccator and store at room temperature.

Created by: Sabia Taqvi
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Protocol for HSC => B/T Cell Differentiation

Steps:

1. **Cell isolation (bone marrow, fetal liver)**
2. **Magnetic cell sorting (CD117, Sca-1, Lin depletion)**
3. **Flow cytometry check of cell sorting**
4. **Coating of plates (antibody?, antigen)**
5. **Cell seeding and medium change**
6. **Flow cytometry analysis**

Starting Populations:

1. CD117+Sca-1hi 4-6 week old mice femurs
2. CD24lo/lin-/CD117+/Sca-1 hi FL cells from timed pregnant mice Swiss.NIH mice;

HSC Sources:

1. 4-6 week old mice femurs
2. Day 15 FL cells from timed pregnant mice Swiss.NIH mice

I. Cell Isolation from Bone Marrow in Mice Femurs:

OBJECTIVE: To isolate cells from the bone marrow of mice femurs

**** In advance, reserve room, make sure supplies are sterilized, prepare ARC box, prepare hood for cell removal.**

ARC Supplies
70% ethanol (spray bottle)
Kimwipes (11x17)
Paper towels
Foam box with ice (for storage of cold PBS)
Cold PBS
Autoclaved scissors
Autoclaved forceps
Killing box
Access card
Lab coat
Gloves
Diapers

Lab Supplies (prepare this and store under UV light in hood before u go and kill mice and isolate femurs)
Have a blue sheet with the following supplies
100ml Beaker with 70% ethanol
5ml Syringes
100ml Beaker for PBS
25 gauge needles (5)
sterile scissors
sterile forceps
50ml centrifuge tube for cells
Put PBS bottle in hood when ready for use (leave in water bath)

Bone Marrow from Femurs Protocol

1. Make sure room is reserved day in advance.
2. Wear lab coat and gloves
3. Spread out diapers and set up table for surgeries. Take out supplies (ethanol, scissors, forceps, napkins, foam box with cold PBS) out of box and place on table. Put paper towels in one hole of killing box and paper towels on bottom of box.
4. Isolate specific mice from Rm 1.1118 and put in killing box
5. Kill using CO2 tank. Only turn bottom gray knob and leave air on for about 5 minutes.

6. Do this for each mice. Spray mouse down with 70% ethanol. Cut off skin near pelvis and peel down to minimize hair in flesh. Cut below patella and put foot, etc. in waste pile. Near the last vertebra in the back of the mouse, make an incision and cut entire leg off. Make sure socket is still sealed. Remove all excess flesh and place femur in PBS tube. Spray ethanol on supplies as necessary (after all skin is removed, etc.)
7. Collect dead mice and parts in napkins/diapers and place in mice body freezer. Clean up table/bench with ethanol and place objects back in container.
8. Wash soiled utensils with water and autoclave in fresh paper. Store in dryer after autoclave.
9. Throw out ice and put ethanoled PBS container with femurs in hood (first turn off UV and turn on light).

Hood Section

10. Ethanol PBS bottle, gloves, timer.
11. Pour 40ml of PBS into 100ml beaker. Open 5ml syringe and load 25 gauge needle. Load barrel with 5ml of PBS from beaker. Lay syringe in beaker ready for use.
12. Remove femur from centrifuge tube and clean all excess flesh not removed earlier using scissors and forceps. Make sure u do not expose the inside of the femur.
13. Place cleaned femur in 70% ethanol for one minute using forceps to hold femur.
14. Cut off tips of femur.
15. Insert syringe needle into one side of femur and position bottom of femur over 50ml centrifuge tube. Slowly flush PBS through femur and wash out so bone appears white. Edges may be a little tricky (cells collect here).
16. Repeat procedure for all femurs and reload syringe with fresh PBS as necessary.
17. Throw away napkin, waste, femurs, syringes, into red autoclave bag. Throw away needles into sharp box. Autoclave soiled scissors and forceps. Store tube with cells in foam box with ice until cell separation.
18. You are now ready for magnetic cell separation.

?????Procedure Regarding FL cell isolation => UNKNOWN!

II. Magnetic Cell Separation (Miltenyi Biotec – lineage depletion, Dynal Biotec – ckit+, sca-1+ isolation)

OBJECTIVE: To isolate lin – ckit+ sca1+ cells using magnetic beads. Will use Miltenyi Biotec system for lin depletion and Dynal Biotec for positive selection.

****Prior to work, store antibodies on ice (make sure enough for expt and check expiration date), prepare buffer, store columns, magnets in hood for sterilization, count cells.**

Example of required calculation:
Each mouse gives 30 x10^6 cells. HSCs can be from 0.01% to 0.1% of original population. (after lineage depletion, ckit isolation, etc.)

THUS,
5 mice x ((30x10^6 cells)/mice = 150 x 10^6 or 1.5 x 10^8 cells
1.5 x 10^8 x 0.01% = 15000 HSCs
1.5 x 10^8 x 0.1% = 150000 HSCs
Range = 15000 – 150000 HSCs

Actual amount of antibodies and buffer will depend on cell count. For example,
For 15 x 10^7 cells, we will need: (these amounts correspond to steps listed below)

40ul x 15 = 600ul of buffer to resuspend cells in
10ul x 15 = 150ul biotinylated antibody
30ul x 15 = 450ul buffer
20ul x 15 = 300ul streptavidin microbeads
10-20x labeling volume => 6 – 12 ml of buffer before spinning
500ul x 1.5 = 750ul buffer added right before magnetic separation

Components Supplied in kit:
1 mL Biotin-Antibody Cocktail: Cocktail of biotin-conjugated monoclonal antibodies CD5, CD45R (B220), CD11b, anti- Ly-6G (Gr-1), 7-4 and Ter-119.
2 mL Anti-Biotin MicroBeads: MicroBeads conjugated to a monoclonal Anti-Biotin antibody (clone: Bio3-18E7.2; mouse IgG1).

Reagents and instruments required:
Buffer (degassed): PBS (phosphate buffered saline without Ca2+ and Mg2+) pH 7.2, supplemented with 0.5% BSA (bovine serum albumin). Keep
buffer cold (4-8 °C).

MACS Columns and MACS Separators: (MS 107 max number of labeled cells 2x108 max number of total cells; LS 108 max number of labeled cells 2x109 max number of total cells; **we will need LS)**
Foam box with Ice for binding
Two 50ml centrifuge tube (for waste, sample)
Four 1 ml centrifuge tubes for cell count, flow cytometry (before and after lineage depletion)
40um nylon mesh filter

Protocol

Preparation of bone marrow cells
All steps should be performed on ice.
1. Disaggregate cells in 50ml tube by gentle pipetting them several times.
2. Pass cells through 30 μm nylon mesh to remove cell clumps. Wet filter with buffer before use.
3. Wash cells by adding buffer, centrifuge at 300xg for 10 minutes at 4–8 °C. Pipette off supernatant completely.
4. Resuspend cell pellet in 5ml of buffer and take an aliquot for cell counting.
5. Cell count: Remove 10μl of cells and place in 1ml ucentrifuge tube. Add 10μl of trypan blue. Mix and add 10μl solution to each side of hemacytometer. Count and note cell amount (amount x 2 x number of mls x 10^4).
6. Calculate amounts of buffers and antibodies needed.
7. Flow: Remove 50μl of cell solution for cell staining and place in 1ml centrifuge tube. Store on ice.

Magnetic labeling
* Fast, pre-cooled cell and antibody solutions will prevent capping of antibodies on the cell surface and non-specific cell labeling.
* When working with less than 107 cells, use the same volumes as indicated. When working with higher cell numbers, scale up all reagent volumes and total volumes, accordingly.
* Working on ice may require increased incubation times. Higher temperatures and/or longer incubation times lead to non-specific cell labeling.

1. Determine cell number.
2. Centrifuge at 300xg for 10 minutes. Pipette off supernatant completely.
3. Resuspend cell pellet in 40 μL of buffer per 107 total cells.
4. Add 10 μL of Biotin-Antibody Cocktail per 107 total cells.
5. Mix well and incubate for 10 minutes at 4-8 °C.
6. Add 30 μL of buffer per 107 total cells.

7. Add 20 μL of Anti-Biotin MicroBeads per 107 total cells.
8. Mix well and incubate for additional 15 minutes at 4-8 °C.
9. Wash cells with buffer by adding 10-20 x labeling volume and centrifuge at 300xg for 10 minutes. Pipette off supernatant completely.
10. Resuspend up to 108 cells in 500 μL of buffer.
11. Proceed to magnetic separation.

Magnetic separation
Note: When working with bone marrow of normal mice, the number of labeled cells is almost equal to the number of total cells. Do following steps in hood. Ethanol columns, magnets, antibody bottles before hood use. Since we are doing lineage depletion we will be using LS columns, large amount of cells.

1. Place LS column in the magnetic field of LS MACS Separator.
2. Prepare LS column by rinsing with appropriate amount of buffer: LS: 3 mL. Place centrifuge tube under magnet ready to collect wash. Place another tube near magnet to get actual sample.
3. Apply cell suspension onto the column. Allow the cells to pass through and collect effluent as fraction
with unlabeled cells, representing the enriched lineage negative cell fraction.
4. Wash column with appropriate amount of buffer. Perform washing steps by adding buffer three times, each time once the column reservoir is empty. LS: 3x3 mL Collect the effluent in the same tube as effluent of step 3. This fraction represents the enriched lineage negative cells.
5. Throw away waste. Remove magnet from inside of hood.

Evaluation of lineage negative cell purity
1. The purity of the enriched lineage negative cells can be evaluated using flow cytometry. Save small aliquot (10ul) for cell count and (50ul) cell purity analysis using flow cytometry.
2. Keep samples on ice (sample in 50ml centrifuge tube, 1ml tube for flow, 1ml tube for cell count). Label tubes so no mixup occurs.
3. Count cells using above procedure prior to positive selection part.

**At this point u will have three tubes on ice. One 50ml centrifuge tube with samples. Two tubes of 50ul cell suspension before and after magnetic cell separation. Before this upcoming part, spin down cells in buffer and resuspend in 1ml.

**Prior to magnetic cell separation, u will need to prepare buffers (DNAase buffer and aliquot this in 10ul portions, PBS with 0.1% Tween 5ml, PBS with 0.1% BSA 5ml, alphaMEM with 1% heat-inactivated (for 30 min at 56C) FBS 5ml).

** Where it says RPMI substitute with alphaMEM.

Dynal Biotech Magnetic Cell Separation (ckit+ sca-1+ cells)

Materials supplied in kit

Component
CELLection™ Dynabeads. Supplied as 4 x 10^8 beads/ml in phosphate buffered saline (PBS), pH 7.4, containing 0.1% bovine serum albumin (BSA) and 0.02% sodium azide ($NaNH_3$)
Releasing Buffer. Component 1. DNase (15,000 – 20,000 U freeze-dried per vial)
Releasing Buffer. Component 2. Buffer

Additional materials required
Biotinylated antibodies ckit/CD117 and sca-1/Ly6AE. (Both from eBiosciences)
Magnetic particle concentrator (Dynal MPC®).
Mixing device allowing tilting and rotation of tubes (Dynal Sample Mixer).
Buffers. (releasing buffer and PBS with 0.1% tween)
Tubes, glass ware, pipettes.
2 - 8°C incubator or ice.

Use at least 25 μl Dynabeads (10^7 beads) and 4 μl Releasing Buffer per ml of sample. For counted samples, to calculate number of Dynabeads to use, Dynal recommends using at least 10^7 beads per ml of sample and the ratio of beads to target cells should be at least 5:1.

Example calculation of bead amount, releasing buffer necessary:

(Consistent with above numbers) We have 150000 cells or 0.15 x 10^6. We have resuspended the cells in 1 ml of PBS with 0.1%Tween. Thus,

25ul of Dynabeads

4ul of Releasing Buffer

As a check step: 150000 x 5 = 750000 beads necessary. The bead suspension comes in 4x10^8 beads/ml. Thus, we need at least 1.875 or 2μl.

Protocol

Perform all steps in hood.

(Do this beforehand) Preparation of Releasing Buffer
Transfer 320 μl buffer (Component 2) to each vial of freeze-dried DNase (Component 1). Dissolve the enzyme gently. Aliquot the Releasing Buffer into 10μl portions and store at -20°C for up to 6 months. Avoid freezing - thawing more than twice per aliquot.

NOTE:
Do not stir the dissolved DNase solution (Releasing Buffer) vigorously at any point in the procedure to ensure a fully active enzyme.

B) Dynabeads washing procedure
Dynabeads should be washed before use. The washing procedure is facilitated by the use of the magnetic device (Dynal MPC).

NOTE:
Serum or protein fractions from serum (e.g. serum albumin) may contain free biotin and should NOT be included in buffers used before and during coupling of biotinylated antibodies to the beads.

1. Resuspend the Dynabeads thoroughly and transfer the desired amount of beads (25 μl) to a tube suitable for the Dynal MPC.
2. Place the tube in Dynal MPC for 1 minute. Make sure the solution is clear and pipette off the fluid without disturbing the beads.
3. Remove the tube from the Dynal MPC, add 1 - 2 ml buffer (e.g. PBS with 0.1% tween) and resuspend.
4. Place the test tube in the Dynal MPC for 1 minute, remove the buffer and take the tube out of the Dynal MPC.

Resuspend the washed Dynabeads back in 25 μl buffer ($4x10^8$ beads/ml).

C) Dynabeads Coating Procedure
The amount of Ab must be titrated for optimal cell yield. The type and the amount of antibody required for the optimal performance of the beads will vary with the Ab-affinity and antigen density on the cell surface.

1. Resuspend washed Dynabeads well by thoroughly shaking the tube.
2. **Add 0.2 - 2 μg of biotinylated antibody per 10^7 beads** directed against an epitope on the desired target cell. 10^7 beads is 25 μl at the supplied concentration.

A concentration of beads in PBS with 0.1% tween from 1-4x10^8 beads/ml is suitable for optimal coating.

3. Rotate the bead and antibody suspension for 30 minutes at room temperature. Would orbital shaker be fine?

4. Place the tube in a Dynal MPC for 1 minute. Make sure the solution is clear and pipette off the fluid.

5. Remove the tube from the Dynal MPC and add 1ml PBS with 0.1% tween.

6. Repeat steps 4 and 5 three times.

7. Resuspend coated beads in original volume of PBS with 0.1% bovine serum albumin (BSA) giving a final concentration of 4 x 10^8 beads/ml. 0.02% sodium azide may be added as a preservative.

The beads are now ready for direct selection of cells from a cell suspension.

D) Isolation of target cells

NOTE:
During incubation and separation procedures it is important to keep the cell suspension and buffers cold (2 - 8°C) to prevent attachment of phagocytotic cells to Dynabeads.

NOTE:
Precoated Dynabeads stored for more than two weeks should be washed once in PBS with 0.1% BSA before use.

1. Prepare the cell sample (e.g. resuspend total cells at 5 - 20x10^6 cells/ml, generally not more than 2x10^6 target cells/ml) and cool it to 2 - 8°C. Cell suspensions prepared from tissue samples can be used but cells must be washed if DNase was used in the tissue digest. When isolating cells from whole blood or buffy coat, wash the sample once (e.g. 10 ml sample plus 40 ml PBS with 0.1% BSA. Centrifuge 800g for 10 minutes. Remove supernatant to the original volume, 10 ml). All that we can ignore, already have it accurate concentration.

2. Resuspend the precoated Dynabeads thoroughly and transfer the desired amount of beads to a tube (25 μl per ml sample).

3. ****????Remove the buffer from the beads and add 1 ml of cell suspension so that the bead concentration is at least 10^7 beads/ml cell suspension and bead:target cell ratio 5:1. Mix cells and beads by light whirlmixing.

4. Place a cap on the tube and incubate for 15 minutes at 2 - 8°C with gentle tilting and rotation (in shaker that's in fridge).

5. Place the tube in the Dynal MPC and leave it to separate for at least 1 minute.

6. Discard the supernatant gently with a pipette while the rosetted cells are attached to the tube wall by the Dynal MPC.

7. Remove the tube from the Dynal MPC and add RPMI 1640 with 1% FCS to the rosetted cells. Use 500 μl for up to 10^8 beads. Increase the volume to 1 ml for up to $2x10^8$ beads and so on.

8. Resuspend the rosetted cells by pipetting and transfer them to a new vial. To increase recovery the first tube can be flushed with another 500 μl medium.

9. Place the tube in the Dynal MPC for 1 minute and pipette off the fluid.

10. Remove the tube from the Dynal MPC and resuspend gently by pipetting the rosetted cells in minimum 500 μl RPMI with 1% FCS

11. Repeat steps 9 and 10 two times.

12. After the final wash, remove the fluid and resuspend the rosetted cells gently in RPMI with 1% FCS pre-warmed to 37°C. Use 200 μl for up to 10^8 beads. Increase the volume to 400 μl for up to $2x10^8$ beads and so on.

13. Add 4 μl of Releasing Buffer for up to 10^8 beads used. Increase the volume to 8 μl for up to $2x10^8$ beads and so on.
14. Cover tube and incubate for 15 minutes at room temperature with gentle tilting and rotation on orbital shaker. Due to the relatively small sample volume, care should be taken that the cells remain in the bottom of the tube during agitation.
15. Flush rosettes vigorously through a pipette 6 – 8 times, then place the tube in the Dynal MPC, leave for 1 minute and pipette the supernatant containing the released cells to a new test tube containing 200 μl RPMI with 10% FCS. Failure to pipette the sample will affect cell yield.
16. Remove the tube from the Dynal MPC.
17. To obtain the residual cells, add 200 μl RPMI with 1% FCS and flush the suspension 4 - 5 times thoroughly through a narrow tipped pipette until no lumps are seen. Place the test tube in the Dynal MPC for 1 minute and transfer the suspension to the same tube as in step 15.
18. The released cells will then be in a total volume of 600 μl. Count cells using hemacytometer. Keep sample on ice. This sample will be used for cell culture.
19. Place 10ul of final cell suspension in a microcentrifuge tube on ice for flow cytometry analysis.

Repeat entire procedure for sca-1 antibody.

Flow Cytometric Analysis
Take 10μl of final cell suspension and use for flow cytometry analysis. Staining selected cells with the same antibody as used for isolation may be performed, but must be done before addition of Releasing Buffer.

???? Would this be us???
Indirect isolation of target cells
For some antibodies and target antigens (especially low antigen expressing target cells) it can be more effective to perform an indirect technique.

1. Incubate cell sample with biotinylated antibody. Use excess antibody (e.g. 1 μg / 10^6 target cells) and incubate for 30 minutes at 2–8°C.

2. Collect pretreated cells by centrifugation at 300g for 10 minutes. Discard the supernatant.

3. Resuspend and wash the pretreated cells once in buffer (HBSS, RPMI or PBS) to remove all unbound antibody. Do not use buffer containing BSA or other serum fractions. BSA with very low biotin should be used if protein is required (PBS with 0.1% BSA).

4. Add washed CELLection™ Dynabeads to the sample (>1 x 10^7 beads per ml sample) to capture the antibody coated cells. Continue from point 3 of procedure above.

Technical Tips
To ensure the most efficient cell capture

1. Titrate the antibody concentration used for coating the CELLection™ Dynabeads.

2. Always use buffers with 0.1% tween and not BSA before the antibody is coated onto the beads to avoid free biotin that may be contained in BSA.

3. The effectiveness of cell separation is dependent upon the effectiveness of the primary antibody. If the biotinylated antibody does not capture the cells it may be due to the biotin molecule affecting the antigen binding site. If this occurs, it is recommended to try an alternative biotinylation method.

To ensure the most efficient cell release

1. Never vortex DNase when resuspending freeze-dried form in 320 μl buffer.

2. After cells are incubated with Releasing Buffer it is essential that the bead / cell complexes are vigorously pipetted before the magnetic separation to provide

mechanical disruption to the DNA linker. Failure to pipette the cells will affect cell yield.

Buffers/solutions

Phosphate buffered saline (PBS) pH 7.4,
NaH_2PO_4 x H_2O 0.16 g
Na_2HPO_4 x 2 H_2O 0.98 g
NaCl 8.10 g
Distilled water to 1 litre

PBS with 0.1% tween
Add 0.1% (v/v) tween-20 to PBS

PBS with 0.1% BSA:
Add 0.1% (w/v) bovine serum albumin to PBS.

RPMI with 1 or 10% heat inactivated FBS for 30min at 56C:
Add 1 or 10% (v/v) fetal calf serum to RPMI 1640.

We're going to use alpha-MEM instead. Should give same results as long as divalent cations are present which they are.

PRECAUTIONS

Resuspend the Dynabeads well before use to obtain a homogeneous dispersion of beads in solution.
Precautions should be taken to prevent bacterial contamination of the product.
Preservatives should be carefully removed before use by washing.
Antibody coated Dynabeads products stored for more than two weeks should be washed once in PBS with 0.1% BSA before use.

III. Flow cytometry check of sorted cells

OBJECTIVE: To check the efficiency of magnetic cell separation procedure of ckit+ sca-1+ cells using flow cytometry.

We will have 4 microcentrifuge tubes of flow cytometry samples:
Before lineage depletion
After lineage depletion
After ckit+ selection
After sca-1+ selection

Supplies
PE-conjugated ckit antibody
FITC-conjugated sca-1 antibody
Isotype controls (PE, FITC)
Fc Block
FACS Buffer (PBS, 1% BSA)
FACS tubes

For each sample, do the following:

1. Fill each tube to 1ml mark with FACS buffer
2. Spin down each tube in microcentrifuge at 1000rpm for 10 min
3. Throw out supernatant and repeat steps 1 and 2.
4. Throw out supernatant and resuspend latter three tubes in 500ul of buffer. For the first tube, suspend in 1.5ml if possible. Then, put 500ul of suspension in one tube and then 500ul into another tube. These will be for the isotype controls (FITC, PE).
5. Add 0.5-1μl of block to each tube and incubate on ice in box for 10 min.
6. Add 0.5-1μl of PE and FITC conjugated antibodies to each tube except the isotype controls. For isotype controls, add 0.5μl of PE isotype control to one tube and 0.5ul of FITC isotype control to the other. Let tubes sit on ice in box for 1 hr.
7. Resuspend cells in tubes after an hour.
8. Spin down at 1000rpm for 10min.
9. Remove supernatant and resuspend cells in fresh buffer (500μl). Spin again at above rate.
10. Remove supernatant and resuspend in 250μl or less. Put suspension in labeled FACS tubes and keep tubes on ice.
11. Proceed to FACS machine and follow appropriate procedure.

IV. DLL4 Coating of Plates

OBJECTIVE: To coat 24 well tissue culture plates with DLL4 at 1.5ug/ml

****This will need to be started right after bone marrow cells have been isolated. Magnetic separation will probably take 3 hours, thus these two tasks can be done in parallel.**

Supplies
24 well tissue culture plates
DLL4 (thaw this before use)
PBS (thaw this before use)
Microcentrifuge tube

Required Calculations:
For 3 wells of DLL4 coating –
1.5ug/ml * .1ml/well * 3 wells = .45ug
.45ug/10ug/ml = 45μl of DLL4

Total Coating liquid => 3 x 100μl = 300μl
300μl – 45μl = 255μl of PBS

45μl of DLL4
255μl of PBS

Protocol

1. Calculate as shown above for desired concentration, well amounts.
2. Add calculated amounts of DLL4 and PBS to microcentrifuge tube.
3. Add 100μl of above prepared solution to each well. Do this fast so protein mixture does not coat centrifuge tube.
4. Incubate at 37C for 2-3 hours to coat the plate.
5. Wash the plate 3 times with PBS (100μl /well) and decant. Only do washing step RIGHT B4 you are ready to seed cells onto coating. In other words, keep coating wet at all times.

** Will need to eventually add in avidin, blocking and antibody steps.

V. Cell Seeding and Maintenance

OBJECTIVE: to seed HSCs onto DLL4 layers in tissue culture plates and differentiate cells to B and T cell lineages with exogenously added cytokines and environmental signals.

****If you look at Hozumi's protocols (with inserts, FL cells), they use 10000 FLCs/ well. If u look at Pflucker's papers (without inserts, HSCs), they use 4000 HSCs/well. Which are we using???**

****Appropriate controls should be put in place. Negative control: HSCs without growth factors, without OP9. Variants: HSCs with OP9 (insert), without growth factors (different combinations).**

Supplies
DLL4 coated 24 well tissue culture plates
SCF
IL-7
Flt-3l
Differentiation medium (alphaMEM, pen-strep, 20%FBS, 2.2g/L NaHCO3)
Lin – Ckit+ Sca-1+ HSCs in tube on ice
PBS
Trypsin
OP9 cells
15ml centrifuge tube

Protocol
1. Prior to HSC work, OP9 cells will have to be trypsanized and counted. Standard procedure should be followed. Old medium aspirated. Cells washed with PBS. Trypsin added to cells and cell incubated at 37C for 10min. Cells washed off using medium. Cells suspended well in medium and 10μl removed for counting. Count using hemacytometer. Cell suspension spun down at 1000rpm for 5 min. Supernatant aspirated. Need 5000 OP9 cells in each well insert. Remove inserts and put in other well positions. Resuspend cells like described below so that 300μl or less = 5000 OP9 cells. Add medium so that there is near 350μl in each insert.
2. HSCs will be suspended in near 600μl of alpha MEM from previous sorting step. We need 10000 cells in each well at 1ml or less volume that is to say, each well cannot supercede 1ml of medium/cell suspension or it will start overflowing. Thus, the suspension concentration should be noted so that each volume is under 1ml. The lowest concentration of cells can be 10000cells/ml (which is really lo conc). SO, if we have 100000 cells total, we should suspend them in 1ml so that 100μl is equal to 10000 cells. The previous cell suspension from sorting step should be spun down in centrifuge at 1000rpm for 5min.

3. Remove supernatant and resuspend in above described way.
4. Wash DLL4 coated wells with PBS 3x.
5. Put appropriate amount in each well and add differentiation medium if necessary to have 1ml volumes in each well.
6. Add growth factors to all wells: SCF, IL-7 (these first 2 are used by Hozumi), Flt-3L (this is consistently used by Pflucker). The following describes the amounts chosen (on a per well basis):
IL-7 (10ng/ml) => 10ng/ml*1ml = 10ng; 10ng/10ug/ml = 1μl
SCF (50ng/ml) => 50ng/ml*1ml = 50ng; 50ng/50ug/ml = 1μl
Flt-3L (20ng/ml) => 20ng/ml*1ml = 20ng; 20ng/5ug/ml = 4μl
7. Replace inserts onto wells. Be careful not to spill medium.

Done for now. Put reagents back in appropriate locations. Seed remaining OP9 cells at low density on TC plate for subsequent experiments.

** After 3 days, aspirate medium. Replace with fresh medium and fresh growth factors in above described amounts.

VI. Flow cytometry of differentiated cells

OBJECTIVE: To analyze the immunophenotype of differentiated cells, specifically for B and T cell lineages after 1wk.

Supplies
CD19-Biotinylated antibody
FITC-IgM (???? Is this necessary? We know that IgM comes later at 1 week)
Streptavidin-PE
CD3-FITC
CD4-PE
CD8-APC
Isotype controls (B, PE, FITC, APC)
Fc Block
FACS Buffer (PBS, 1% BSA)
FACS tubes

**Nitty gritty of isotype controls need to be added. Need to assign appropriate controls to PE, FITC, B, APC conjugated antibodies.

Protocol
For each sample, do the following:

1. After 1 wk, samples from well need to be removed. Cells will have to be trypsanized and counted. Standard procedure should be followed. Old medium aspirated. Cells washed with PBS. Trypsin added to cells and cell incubated at 37C for 10min. Cells washed off using medium. Cells suspended well in medium and 10ul removed for counting. Count using hemacytometer.
2. Separate samples into separate centrifuge tubes. Each sample should be separated into isotype controls, single positive and double positive stained cells.
3. Fill each tube to 1ml mark with FACS buffer
4. Spin down each tube in microcentrifuge at 1000rpm for 10 min
5. Throw out supernatant and repeat steps 1 and 2.
6. Throw out supernatant and resuspend tubes in 500ul of buffer.
7. Add 0.5-1μl of block to each tube and incubate on ice in box for 10 min.
8. Add 0.5-1μl of Biotinylated, PE, FITC, APC conjugated antibodies to tubes except the isotype controls. For isotype controls, add 0.5μl of PE isotype control, 0.5μl of FITC isotype control, 0.5μl of APC isotype control. Let tubes sit on ice in box for 1 hr.
9. Resuspend cells in tubes after an hour.
10. Spin down at 1000rpm for 10min.
11. Remove supernatant and resuspend cells in fresh buffer (500μl). Add PE-Streptavidin to biotinylated-CD19 samples. Let tubes sit on ice in box for 1 hr.
12. Spin again at above rate.
13. Remove supernatant and resuspend cells in fresh buffer (500μl). Spin down again. Resuspend in 500μl. Put suspension in labeled FACS tubes and keep tubes on ice.
14. Proceed to FACS machine and follow appropriate procedure.

Important Abbreviations

APC = antigen presenting cell
BMHSC/BMHSCs = bone marrow hematopoietic stem cells
CD_ = clusters of differentiation (cell surface markers)
CMV = cytomegalovirus
DCs = dendritic cells
DGS = Di George's Syndrome
DL1/DLL1 = Delta like ligand 1
DL4/DLL4 = Delta like ligand 4
DN = double negative
DP = double positive
EB = embryoid body
ECM = extra-cellular matrix
EGF = epidermal growth factor
ESC/ESCs = embryonic stem cell
ETPs = Early T lineage progenitors
FACS = fluorescence associated cell sorting
FTOCs = fetal thymic organ culture
HERP2 = HES1 related protein gene
HES1 = hairy enhancer of split gene
HIS = histidine
HLA = human leukocyte antigen
HPC/HPCs = hematopoietic progenitor cells
HSC/HSCs = hematopoietic stem cells
ICN = intracellular Notch
IL-7 = interleukin 7
MFI = mean fluorescence intensity
MHC = major histocompatibility complex
MSEF = mean soluble equivalent fluorophores
NK = natural killer cells
PLGA = poly lactic glycolic acid
PLLA = poly L lactic acid
PMC = perimedullary cortex
QSC = quantum simply cellular kit
RTOCs = reaggregate thymic organ culture
SCF = stem cell factor
SP = single positive
TCR = T cell receptor

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Vita

Sabia Zehra Taqvi is the daughter of Zafar and Nasim Taqvi. She was born in Houston, Texas on September 6th 1980 and has two younger siblings, Nazer and Sarah. She attended elementary, intermediate and high schools in Houston and pursued her interest in science and math at Texas A&M University in College Station, Texas. Sabia graduated with honors with a Bachelor's in Science degree in biomedical engineering and a minor in Business Administration in the fall of 2002.

Sabia continued her education with graduate studies in biomedical engineering at the University of Texas in Austin in the fall of 2002. Her doctoral studies centered on *ex vivo* systems for hematopoietic differentiation applications with a special focus on T cell differentiation using Notch ligand functionalized microbeads. Sabia's efforts led to a provisional patent, several national conference presentations and two first author publications in peer-reviewed journals. Sabia is a NSF IGERT fellow, NIH Biotechnology trainee and a recipient of Thrust Engineering Graduate Fellowship. She was also recognized for her service as a teaching assistant. In June of 2006, Sabia was married to Wasif Ali Abidi, a M.D./Ph.D. student at New York University. Both Wasif and Sabia's immediate and extended families have played an instrumental part in her success and well-being. Sabia intends to give back to the community through a teaching career and initiatives in scientific education.

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